Diagnostic markers of clinical allergy versus sensitisation to peanut

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DIAGNOSTIC MARKERS OF CLINICAL ALLERGY VERSUS SENSITISATION TO PEANUT

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To my Parents

To the memory of my Grandfather Mário Figueira

Abstract

Peanut-specific IgE is necessary but not sufficient to elicit allergic reactions to peanut. Oral food challenge, the gold-standard for the diagnosis of peanut allergy, is indicated when tests based on the presence of IgE are equivocal; however, this is resource-intensive and carries the risk of an allergic reaction.

My aims were to improve the diagnosis of peanut allergy and our understanding of the discrepancy between the presence of IgE (i.e. allergic sensitisation) and clinical allergy to peanut. Peanut allergic, peanut-sensitised but tolerant and non-sensitised non-allergic subjects were studied. Participants underwent clinical evaluation, skin prick testing, blood collection for serology and basophil and mast cell assays and oral food challenge to peanut, if clinically indicated.

The basophil activation test had 97% accuracy to diagnose peanut allergy and reduced the need for oral food challenges by 66%. It proved to be particularly useful in cases in which conventional allergy tests failed to diagnose peanut allergy. The basophil activation test also estimated the severity and the threshold of allergic reactions to peanut.

Peanut allergic patients had higher levels of peanut-specific IgE and were more likely to have IgE to peanut major allergens. Peanut-sensitised but tolerant patients showed a predominance of IgG4 over IgE. Their plasma inhibited peanut-induced activation of mast cells and basophils *in vitro* similar to plasma from patients submitted to peanut oral immunotherapy. The role of IgG4 in the inhibition of peanut-induced effector cell activation was confirmed by depletion of IgG4 from plasma samples of tolerant patients sensitised to major peanut allergens, which would otherwise be predictors of peanut allergy.

In conclusion, the basophil activation test reproduced *in vitro* the clinical phenotype of peanut-sensitised patients. Characteristics of IgE and the presence of IgG4 and possibly other blocking antibodies are two non-mutually exclusive explanations for the discrepancy between peanut allergy and sensitisation.

Publications related to this work

1.1 Original articles

1. George Du Toit, Graham Roberts, Peter H. Sayre, Henry T. Bahnson, Suzana Radulovic, Alexandra F. Santos, Helen Brough, Deborah Phippard, Monica Basting, Mary Feeney, Victor Turcanu, Michelle L. Sever, Margarita Gomez, Marshall Plaut, Gideon Lack, for the LEAP Study Team. **The LEAP randomized control trial: prevention of peanut allergy through early consumption in high-risk infants.** New Engl J Medicine 2015 (in press)

2. Alexandra F. Santos, Louisa K. James, Henry T. Bahnson, Mohammed H. Shamji, Natália C. Couto-Francisco, Sabita Islam, Sally Houghton, Andrew T. Clark, Alick Stephens, Victor Turcanu, Stephen R. Durham, Hannah J. Gould, Gideon Lack. **IgG4 inhibits peanut-induced basophil and mast cell activation in peanut tolerant children sensitized to peanut major allergens**. J Allergy Clin Immunol 2015 (in press). *Selected as the Editor's choice.*

3. Alexandra F. Santos, George Du Toit, Abdel Douiri, Suzana Radulovic, Alick Stephens, Victor Turcanu, Gideon Lack. **Distinct parameters of the basophil activation test reflect the severity and the threshold of allergic reactions to peanut**. J Allergy Clin Immunol 2015; 135 (1):179-86. *Selected as the Editor's choice.*

4. Alexandra F. Santos, Abdel Douiri, Natalia Bécares, Shih-Ying Wu, Alick Stephens, Suzana Radulovic, Susan M. H. Chan, Adam T. Fox, George Du Toit, Victor Turcanu, Gideon Lack. **Basophil activation test discriminates between allergy and tolerance in peanut-sensitized children.** J Allergy Clin Immunol 2014; 134(3): 645-52. *Published with a dedicated editorial[1] and selected as the Editor's choice.*
5. Yih-Chih Chan, Faruk Ramadani, Alexandra F. Santos, Prathap Pillai, Line Ohm-Laursen, Clare Harper, Cailong Fang, Shih-Ying Wu, Ying Sun, Chris J. Corrigan, Hannah J. Gould. **"Auto-anti-IgE": naturally occurring IgG anti-IgE antibodies may inhibit allergen-induced basophil activation**. J Allergy Clin Immunol 2014; 134 (6): 1394-1401.

6. Helen A. Brough , Alexandra F. Santos, Kerry Makinson , Martin Penagos, Alick C. Stephens, A. Douiri, Adam T. Fox, George Du Toit, Victor Turcanu, Gideon Lack. **Peanut protein in household dust is related to household peanut consumption and is biologically active**. J Allergy Clin Immunol 2013; 132(3):630-8.

7. Alexandra F. Santos, Natalia Bécares, Alick Stephens, Victor Turcanu, Gideon Lack. **CD123 is down-regulated with basophil activation – implications for the gating strategy of the basophil activation test** (submitted).

1.2 Review articles, editorials and position papers

1. Alexandra F. Santos, George Du Toit, Gideon Lack. **Is the use of epinephrine a good marker of severity of allergic reactions during oral food challenges?** J Allergy Clin Immunol: In Practice 2015 (in press).

2. Hans Jürgen Hoffmann, Alexandra F. Santos, Cristobalina Mayorga, Anna Nopp, Bernardette Eberlein, Marta Ferrer, Paul Rouzaiere, Didier Ebo, Maria Luisa Sanz, Tatjana Petcovic, Sarita Patil, Oliver Hausmann, Wayne G. Shreffler, Peter Korosec, Edward F. Knol. **Clinical impact of the basophil activation test in the diagnosis and monitoring of allergic disease**. Allergy (submitted).

3. Graham Roberts, Markus Ollert, Rob Aalberse, Moira Austin, Adnan Custovic, Audrey DunnGalvin, Philippe Eigennman, Filippo Fassio, Clive Grattan, Peter Hellings, Jonathan Hourihane, Edward Knol, Antonella Muraro, Nikos Papadopoulos, Alexandra F. Santos, Sabine Schnadt. **A new framework for the interpretation of IgE sensitization tests: position paper of the European Academy of Allergy and Clinical Immunology**. Allergy (under review).

4. Pascal Demoly, Luciana Tanno, Cezmi A. Akdis, Susanne Lau, Moises A. Calderon, Alexandra F. Santos, M. Sanchez-Borges, L.J.Rosenwasser, Ruby Pawankar, N.G.

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7. Sangeeta Dhami, Sukmeet S. Panesar, Graham Roberts, Antonella Muraro, Margitta Worm, Maria Beatrice Bilò, Victoria Cardona, Anthony E. J. Dubois, Audrey Dunn Galvin, Philippe Eigenmann, Monserrat Fernandez-Rivas, Susanne Halken, Gideon Lack, Bodo Niggemann, Franziska Rueff, Alexandra F. Santos, Berber Vlieg–Boerstra, Zaraquiza Zolpikli, and Aziz Sheikh, on behalf of the EAACI Food Allergy and Anaphylaxis Group. **Management of anaphylaxis: a systematic review**. Allergy 2014; 69(2):168-75.

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9. Alexandra F. Santos, Gideon Lack. **Commentary on the Cochrane Review "Glucocorticoids for the treatment of anaphylaxis"**. Evidence-Based Child Health 2013; 8: 1295–1296.

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List of Abbreviations

A1	Purified Ara h 1
A2	Purified Ara h 2
A3	Purified Ara h 3
aFcεRI	Anti-FcεRI
aIgE	Anti-IgE
ALK	Peanut extract from ALK-Abelló
APC	Allophycocyanin
AUC	Area under the curve
AUC CD63	Area under the dose-response curve considering the %CD63+ basophils
AUC ROC	Area under the receiver operating characteristic curve
BAT	Basophil activation test
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
CD	Clusters of differentiation
CI	Confidence interval
CNBr	Cyanogen bromide
CPE	Crude peanut extract
CPP	Crude peanut protein
DBPCFC	Double-blind-placebo-controlled-food-challenge
EC	Effective concentration
EC_{50}	Half-maximal effective concentration
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
ENPP	Ectonucleotide pyrophosphatase/phosphodiesterase
ERK	Extracellular signal-regulated kinases
FITC	Fluorescein isothiocyanate
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FSC	Forward scatter

GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
HSA	Human serum albumin
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol triphosphate
IQR	Interquartile range
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JNK	c-Jun N-terminal kinase
LAMP	Lysosomal-associated membrane protein
LAT	Linker for activation of T cells
LEAP	Learning Early About Peanut Allergy
LR	Likelihood ratio
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MFI	Mean fluorescence intensity
MIP	Macrophage inflammatory protein
MW	Molecular weight
NA	Non-sensitised non-allergic to peanut
NF-κB	Nuclear factor- κB
NFAT	Nuclear factor of activated T-cells
NHS	N-hydroxysuccinimide
NPV	Negative predictive value
NR	Non-responders
OIT	Oral immunotherapy
OFC	Oral food challenge
p38-MAPK	p38-mitogen-activated protein kinase
PA	Peanut allergic
PAS	Peanut Allergy Sensitisation
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PE	Phycoerythrin
PECy7	Phycoerythrin-Cy7
PerCP	Peridinin chlorophyll protein
pI	Isoelectric point
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKC	Protein kinase C
PPV	Positive predictive value
PR	Pathogenesis-related protein
PS	Peanut-sensitised but tolerant
RANTES	Regulated on activation, normal T cell expressed and secreted
ROC	Receiver-operating characteristic
RPMI	Rosewell Park Memorial Institute medium
RR	Relative risk
SHIP	SH2-containing inositol 5-phosphatase
SHP	SH2-containing protein tyrosine phosphatase
SI	Stimulation index
sIgE	Specific IgE
SPT	Skin prick test
SSC	Side scatter
STOP	Study of Tolerance to Oral Peanut
Syk	Spleen tyrosine kinase
Th	T helper
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
Tr1	T regulatory 1 cell

Chapter 1 Introduction

1.1 Food allergy

Food allergy is part of a larger spectra of clinical entities designated food hypersensitivity or adverse reactions to foods[2, 3]. An adverse reaction to food is considered food allergy when it is mediated by the immune system. Food allergies are classified based on the involvement of IgE antibodies in its pathogenesis in: IgE mediated, mixed IgE and non-IgE mediated and non-IgE mediated food allergies (Figure 1.1). IgE-mediated food allergies are also designated "immediate-type food allergies" as symptoms develop usually within minutes up to one hour following exposure to the food allergens. The manifestations of the other two types of food allergies (non-IgE-mediated and mixed IgE and non-IgE mediated) usually develop more than 2 hours after allergen exposure and/or manifest chronically. Non-immunological adverse reactions to foods are also designated food intolerances and can have a diversity of underlying aetiologies, such as metabolic (e.g. lactose intolerance, fructose intolerance, galactosemia), pharmacologic (e.g. mediated by histamine, tiramine, caffeine or teobromin), toxic (e.g. gastroenteritis, scombroid food poisoning) or other (e.g. pancreatic insufficiency, biliary conditions, hyatal hernia, gustatory rhinitis, auriculo-temporal or Frey syndrome, blepharochalasis).

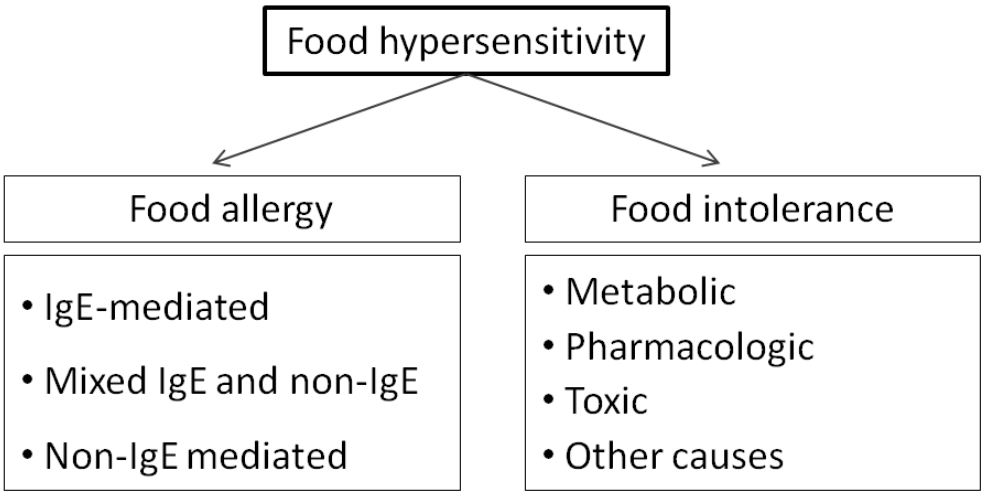


Figure 1.1 Classification of adverse reactions to foods

The clinical presentation of food allergy depends on the underlying mechanism, not only with respect to timing after allergen exposure, but also to the type of symptoms that typically develop. The clinical manifestations of immediate-type food allergies can affect all organs and systems,

including: the skin (for example, pruritus, erythema, acute urticaria, angioedema, contact urticaria), the gastrointestinal tract (for example, vomiting, diarrhoea, abdominal pain, mild oral symptoms such as oral pruritus), the respiratory tract (for example, rhinorrhoea, nasal obstruction, sneezing, sometimes accompanied by lacrimation and ocular pruritus, laryngeal oedema and wheezing) and the cardiovascular system (for example, paleness, hypotension, tachycardia and shock). The most severe clinical presentation of food allergy is anaphylaxis. Anaphylaxis is defined in the new guidelines of the European Academy of Allergy and Clinical Immunology as a "severe, potentially life-threatening systemic hypersensitivity reaction characterized by being rapid in onset with life-threatening airway, breathing, or circulatory problems and is usually, although not always, associated with skin and mucosal changes"[4].

Theoretically, any food can cause allergies but in practice a small group of foods is responsible for more than 90% of immediate-type food allergies[5]. In children, the most common food allergies are allergy to peanut, cow's milk, egg, wheat, soya, tree nuts and seeds, fish and shellfish. In adults, the most common food allergies are: peanut and tree nut allergies, fish and shellfish allergies and allergies to fresh fruits.

1.2 Oral tolerance

Food allergies can be seen as disruption in the physiological process of oral tolerance. Immunologically, oral tolerance is an antigen-specific suppression of cellular and humoral responses to dietary antigens following exposure in the gastrointestinal tract. It is thought to be determined by a combination of factors, including physical factors, antigen dose and timing, microbiome, dendritic cells and other antigen-presenting cells and T cell and antibody responses[6].

1.2.1 Definition of oral tolerance

There is considerable fluidity in the definition of oral tolerance in the literature, especially in the context of food allergy. Clinically, oral tolerance can be defined as the ability to eat the food without developing immunologically mediated symptoms irrespective of the frequency and the quantity of food that is consumed. It is conceivable that such a stringent definition of tolerance can only occur once clonal deletion (i.e. programmed cell death of T cells) or anergy (i.e. failure to activate T cells) has happened, whereas ongoing active suppression of an immune response to

food, for example by regulatory T cells, may require intermittent antigen exposure and thus regular consumption of the implicated food to maintain oral tolerance, but this is still under debate.

1.2.2 Immunological mechanisms of oral tolerance

The mucosal response to protein antigens in early life is typically Th2-skewed and IgE to foods can develop both in atopic and non-atopic infants. In non-allergic children, this IgE response is typically transient and the Th2 response is counterbalanced by a Th1 response leading to tolerance. In mouse models, the exposure to a high dose of antigen, even as a single dose, via the oral route, induced oral tolerance as reflected by the absence of hypersensitivity responses, T cell proliferation, and cytokine and antibody production[7]. It is possible that a similar phenomenon happens in humans during weaning and that early oral exposure to high dose of allergens induces oral tolerance. This is currently being investigated in randomised controlled trials, namely the LEAP study[8]. Such abrogation of response could either result from the absence of immune response (anergy) or from programmed cell death (deletion) of reactive T cell clones. Whether T cell clone anergy or deletion occurs depends on whether the interaction between the TCR and the MHC-class II on antigen presenting cells occurs in the presence of Fas-Fas-ligand interaction or in the absence of co-stimulation. Low dose exposure to the antigen, especially if repeated, induces T regulatory cells, such as CD4+ CD25+ Foxp3+ naturally occurring Tregs (which exerts its suppressive action via cell to cell contact and via the production of TGF-β) and inducible or adaptive Tregs (TR1 which produce IL-10 and Th3 which produce TGF-β). Cytokines produced by Tregs can influence B cells and lead to the production of IgG4 (induced by IL-10) and IgA (induced by TGF-β). Antibodies of an isotype other than IgE, such as IgG4 and IgA, can counteract the function of IgE either by competing for binding to the allergen or by intracellular inhibitory signalling possibly resulting from co-cross-linking of the FcεRI with ITIM-associated receptors such as FcγRIIB or FcαRI. Microbial stimulation provided by the enteric flora through innate immune receptors is critical for the development and organisation of mucosal and secondary gut-associated lymphoid tissue[9] and may play an important role in the modulation of the immune response leading to oral tolerance.

1.3 Epidemiology of peanut allergy

Food allergy affects about 8% of children and 2% of adults in Western countries. Reported food allergy is more common than food allergy diagnosed based on allergy tests alone, which in turn is more common than food allergy confirmed by oral food challenge. The overall prevalence of food allergy in children and adults has been estimated to affect about 12-13% of patients if only reported allergy is considered and about 3% of subjects if only challenge-proven food allergy is considered. The prevalence of food allergy is thought to be increasing and this is best described for peanut allergy[10, 11]. Apart from the recent increase in the prevalence of food allergy, there has also been a change in the clinical spectra of this condition with food allergies manifesting from a very early age, to multiple unrelated foods, with severe manifestations, including anaphylaxis, and with persistence of the allergy until later in life[12].

Peanut allergy is one the most common food allergies in developed countries, particularly in the United Kingdom, the United States and Australia, where its prevalence ranges from 1 to 3%[11, 13-15]. The prevalence of peanut allergy seems to be increasing. Although a report from birth cohort studies performed in the Isle of Wight suggested a recent stabilisation in the prevalence of peanut allergy, with 0.5% of 4-year-olds being affected in 1996, 1.4% of 3-year-olds being affected in 2002 and 1.2% of 3-year-olds being affected in 2008[16], recent figures from the Healthnuts study performed in Australia reported the highest prevalence of challenge-proven peanut allergy at 3% in 12-months old infants[15], which suggests an increase in the prevalence of peanut allergy in the younger generations.

The increase in the hospitalization rates for peanut-induced anaphylaxis seems to follow the increase in the prevalence of peanut allergy[17]. In a retrospective study performed in the United States using the New York State hospitalisation database between 1990 and 2006[17], food anaphylaxis was the most frequent (67.4%) reason for anaphylaxis admissions after 1993, with peanut anaphylaxis constituting the largest proportion of cases (28.9% in 2006). The number of cases of peanut anaphylaxis increased more than the other food groups over time, with a 9-fold increase in the number of admissions from 1990 to 2006. Also in the United Kingdom, the number of hospital admissions for food-induced anaphylaxis from 1998 to 2012 has increased about 106% overall and 137% in the age group from 0 to 14 years old[18]. Although the increase in the hospital admissions has fortunately not translated into increase in food anaphylaxis fatalities, peanut and tree nut allergies still account for the majority of food allergy-related deaths[18-20].

1.4 The impact of peanut allergy

Peanut allergy affects children at a very young age, with up to 80% of cases presenting after their first known exposure to peanut[21]. It is usually persistent, resolving for only 20% of young children by school age[22]. Peanut allergy is often responsible for severe allergic reactions and for a great proportion of food anaphylaxis fatalities[20], being the commonest cause of life-threatening anaphylaxis in childhood[13, 23]. Patients can have a severe reaction on the first time they consume peanut[24]. Oral food challenges, the gold-standard for the diagnosis of peanut allergy, can also cause severe reactions in a significant proportion of cases[25].

There is no curative treatment for peanut allergy and the mainstay of its management currently is strict avoidance of peanut-containing foods and the implementation of an emergency plan to treat accidental allergic reactions, which includes adrenaline auto-injectors in the severe cases. Since peanut is a ubiquitous food and affected patients often react to small doses, avoidance is difficult and accidental allergic reactions are common[26, 27]. Therefore, peanut allergic children and their families and carers experience a poorer quality of life due to food and social restrictions and to the potentially life-threatening nature of this allergy[28]. The quality of life of children with peanut allergy has been found to be more impaired than the quality of life of children with insulin-dependent diabetes[29], for example. Recent studies suggest that peanut allergy can also have a significant psychological impact on the lives of the patients and their families with fear of eating, anti-social behaviour, anxiety and parental stress[28, 30].

1.5 Peanut allergens

Twelve different allergens have been identified in peanut (Table 1.1). Ara h 1[31], Ara h 2[32] and Ara h 3[33] are peanut major allergens. These 3 storage proteins are stable to temperature, acidity and proteolytic digestion and thus are potent allergens, able to induce primary allergic responses to peanut and severe systemic symptoms in allergic individuals. Ara h 4, previously registered as a separate allergen, is currently considered an isoform of Ara h 3 and has been renamed to Ara h 3.02. Belonging to the same protein family as Ara h 2, Ara h 6 and Ara h 7 are also considered "true peanut allergens" and particularly Ara h 6 has been reported as being able to cause severe systemic allergic reactions and as being a major allergen in populations of peanut allergic patients in Europe[32, 34-36]. On the contrary, Ara h 5, a profilin[32], and Ara h 8, a Bet v 1 homologue[37], are

involved in pollen-food syndromes due to cross-reactivity of antibodies primarily directed to pollen allergens, such as grass pollen profilin and birch pollen Bet v 1 respectively, but are not usually responsible for systemic allergic reactions to peanut. Ara h 9, a lipid transfer protein, appears to have different clinical relevance depending on the geographical location, eliciting systemic reactions in Southern European countries[38-40]. Ara h 10 and Ara h 11 are oleosins[41], which are hydrophobic proteins intimately related to lipid structures and can cause severe allergic reactions. Finally, Ara h 12 and Ara h 13 are defensins and are also very stable proteins.

Table 1.1 Peanut allergens grouped by protein family.

Abbreviations: MW, molecular weight; pI, isoelectric point; PR, pathogenesis related protein.

Protein Family	Allergens	MW (KDa)	pI
Cupin (Vicillin-type, 7S globulin)	Ara h 1.0101	68.8	6.4
Cupin (Legumin-type, 11S globulin)	Ara h 3.0101	58.3	5.7
	Ara h 3.0201	61.0	5.5
Conglutin (2S albumin)	Ara h 2.0101	18.0	5.5
	Ara h 2.0201	17.7	5.3
	Ara h 6.0101	14.8	5.5
	Ara h 7.0101	16.3	5.6
	Ara h 7.0201	17.4	7.5
Profilin	Ara h 5.0101	14.1	4.6
PR-10	Ara h 8.0101	17.0	5.0
	Ara h 8.0201	16.4	5.1
Nonspecific Lipid-transfer protein 1	Ara h 9.0101	9.1	9.5
	Ara h 9.0201	9.1	9.3
Oleosin	Ara h 10.0101	17.8	9.6
	Ara h 10.0201	15.5	9.4
	Ara h 11.0101	14.3	10.1
Defensin	Ara h 12.0101	7.9	7.7
	Ara h 13.0101	8.4	7.5

Ara h 1, Ara h 2, Ara h 3 and Ara h 6 are the allergens most commonly recognised by peanut allergic patients' IgE, except for Southern European countries, such as Spain, where Ara h 9 gains more importance[35, 39, 42]. Ara h 2 has shown to be the best discriminator of peanut allergic patients[43-45] and the most potent elicitor of cell activation and mediator release *in vitro*[46, 47].

1.6 Immunologic mechanisms of peanut allergy

The immunologic mechanism underlying peanut allergy is type I hypersensitivity. Following allergic sensitisation, where peanut allergens are presented to T cells and a Th2-skewed immune response commits B cells to IgE production, peanut-specific IgE binds to the high affinity IgE receptors (FcεRI) on the surface of mast cells and basophils. In allergic individuals, on subsequent exposure to peanut, multivalent allergens bind to receptor-bound allergen-specific IgE on the surface of mast cells and basophils which leads to cross-linking of IgE molecules and aggregation of FcεRI receptors. These initiate complex intracellular signalling cascades which culminate in mast cell and basophil activation and degranulation with the release of pre-formed mediators and de novo synthesis of leukotrienes and cytokines, all of which contribute to allergic inflammation and are responsible for the early and late phase symptoms of acute allergic reactions[48, 49].

1.6.1 Intracellular signalling cascade

Following IgE cross-linking by allergen, the signalling cascade downstream of FcεRI[50-55] starts with activation of Lyn, possibly due to de-phosphorylation of its regulatory site by tyrosine phosphatase CD45. Activated Lyn phosphorylates tyrosine residues of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the β and γ chains of FcεRI. Phosphorylated ITAMs serve as docking sites for spleen tyrosine kinase (Syk). Activation of Syk leads to auto-phosphorylation and phosphorylation of downstream proteins, such as the linker for activation of T cells (LAT), Bruton's tyrosine kinase (BTK) and the phosphoinositide 3-kinase (PI3K) pathway (Figure 1.2). Activated phospholipase C hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2) present in the plasma membrane to produce second messengers, including inositol triphosphate (IP_3), which leads to an increase in intracellular calcium, and DAG, which activates protein kinase C (PKC). A number of other intracellular signals are thought to play a role in subsequent events, namely: activation of small GTPases (e.g. Rac, Ras and Rho) leading to activation of extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK) and p38-mitogen-activated protein kinase (p38-MAPK), which function as mediators between the cytosol and the nucleus and regulate transcription factors; granular fusion and release of mediators; activation of calcineurin, which de-phosphorylates nuclear factor of activated T-cells (NFAT) leading to the transcription of cytokine genes; phosphorylation and degradation of the inhibitor iKB, which leads to the release

and nuclear translocation of NF-KB (nuclear-factor kB) proteins and consequent transcription of pro-inflammatory mediators; and, finally, PKC and MAPK action on phospholipase A2 which releases arachidonic acid and leads to prostaglandin and leukotriene production.

FcεRI receptor aggregation also activates negative regulators which limit the intensity and duration of positive signals[56-59]. For example, SH2-containing inositol 5-phosphatase (SHIP) is recruited to the plasma membrane and hydrolyses PIP_3 products, the main activator of PI3K signalling. SH2-containing protein tyrosine phosphatases, SHP-1 and SHP-2, de-phosphorylate other signalling molecules such as tyrosine kinases.

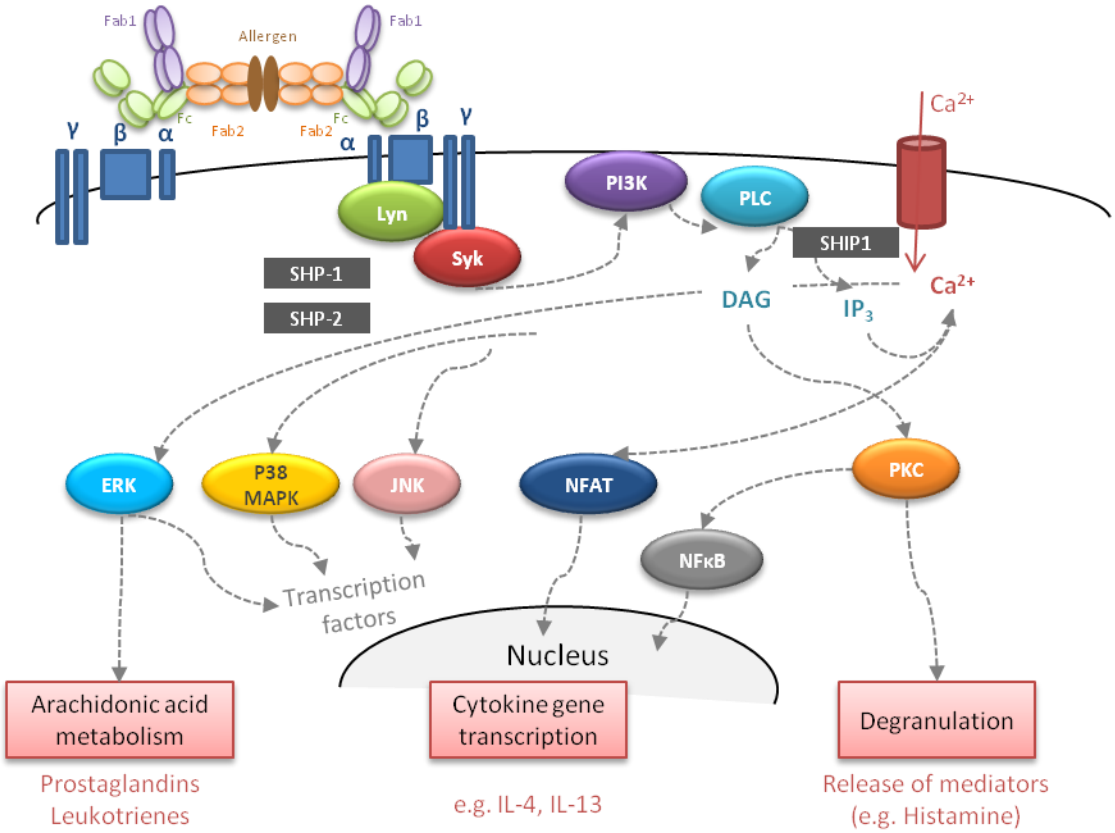


Figure 1.2 Simplified schematic representation of the signalling cascade downstream the high-affinity IgE receptor, FcεRI.

FcεRI is composed of 4 chains: one α chain, one β chain and two γ chains. An allergen cross-linking two IgE molecules, with their Fab and Fc regions, are represented. Syk, spleen tyrosine kinase; PI3K, Phosphoinositide 3-kinase; PLC, phospholipase C; DAG, diacylglycerol; IP3, inositol triphosphate; PKC, Protein kinase C; ERK, extracellular signal-regulated kinases; MAPK, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinase; NFAT, Nuclear factor of activated T-cells; NFkB, nuclear-factor kB; SHIP, SH2-containing inositol 5-phosphatase; SHP, SH2-containing protein tyrosine phosphatase.

1.6.2 Activation and mediator release by basophils and mast cells

Mast cells and basophils can differ in the substances that can elicit cell activation and in the mediators that they release during degranulation (Table 1.2)[49, 60].

Table 1.2 Examples of stimulants of and mediators released by basophils and by mast cells.

*Catepsin G exists in mast cells localised to the skin, submucosae, perivascular tissue and conjunctiva (MCTC) but not in alveolar or epithelial mast cells (MCT). Abbreviations: fMLP, formyl-methionyl-leucyl-phenylalanine; PAF, platelet activating factor; LT, leukotrien; PG, prostaglandin; IL, interleukin; TNF, tumour necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; MIP-1, macrophage inflammatory protein.

	Basophils	Mast cells
Stimulants	• IgE cross-linking (allergens, anti-IgE) • fMLP • C5a • Ionomycin	• IgE cross-linking (allergens, anti-IgE) • Morphine and codeine • Substance P • C5a • Ionomycin
Pre-formed mediators	• Histamine • Chondroitin sulphate • Heparin • PAF	• Histamine • Tryptase • Chondroitin sulphate • Heparin • Chymase • Carboxipeptidase A3 • Catepsin G*
Leukotrienes Prostaglandins	• LTC_4	• LTB_4, LTC_4, LTD_4 • PGD_2
Cytokines	• IL-4 • IL-13	• IL-4, IL-13 • IL-5, IL-6, IL-8, IL-16, TNF-α, GM-CSF, MCP-1, MIP-1-α

1.7 The basophil activation test

IgE-mediated allergic reactions can be reproduced *in vitro* using mast cells and basophils. Basophils have the advantage of being easily available as they can be readily isolated from peripheral blood. Traditionally, functional *in vitro* tests based on allergen-induced activation of IgE-bearing mast cells and basophils were based on the measurement of mediators released by these cells after stimulation with allergen, such as histamine and β-hexosaminidase[61]. However,

during degranulation, together with the release of vasoactive mediators, basophils up-regulate the expression of different activation markers on their surface - Figure 1.3. The expression of these markers, particularly of CD63, correlates with histamine release[62, 63].

The basophil activation test (BAT) is a flow cytometry-based assay where the expression of activation markers is measured on the surface of basophils following stimulation with allergen[60, 64]. A positive basophil activation test can be seen as an *in vitro* surrogate of an acute allergic reaction *in vivo*. In a study of patients allergic to hymenoptera venom, up-regulation of basophil activation markers was observed both *in vitro* following stimulation with yellow jacket or honey bee venom and *in vivo* following a positive sting challenge. In the same study, there was a general agreement between the clinical presentation (systemic reaction *versus* large local reaction) and the results of BAT, suggesting that the BAT is a potential biomarker of anaphylaxis.

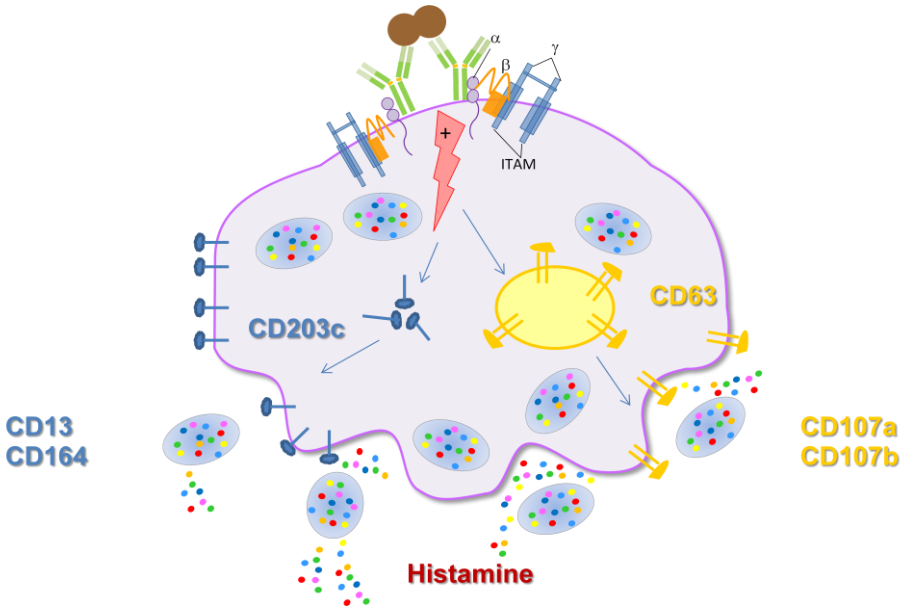


Figure 1.3 Basophil activation markers

Basophil activation markers seem to form two distinct groups that are up-regulated concomitantly: CD63, CD107a and CD107b (in yellow) and CD203c, CD13 and CD164 (in blue). Abbreviations: ITAM, immunoreceptor tyrosine-based activation motif

1.7.1 Basophil identification markers

Different cell-surface markers can be used to identify basophils in whole blood, including IgE, CD123, CCR3, CRTH2, or CD203c[64] (Table 1.3).

Table 1.3 Basophil identification markers

Marker	IgE	CD123	CCR3	CRTH2	CD203c
Synonym	-	IL-3Rα	CD193	CD294	Neural cell surface differentiation antigen
Function	Defence against helminths, type I hypersensitivity	Low-affinity (α) subunit of IL-3 receptor	Receptor for C-C type chemokines	Receptor for prostaglandin D2	Unknown
Peripheral blood cells expressing the marker	Monocytes, dendritic cells, basophils, B cells, and platelets	Basophils, monocytes, eosinophils, plasmacytoid dendritic cells, myeloid dendritic cells, and subsets of haematologic progenitor cells	Basophils, eosinophils, Th2 cells	Basophils, eosinophils, Th2 cells	Basophils
Markers to be used in combination	HLA-DR[1]		CD3[2]		None[3]

[1] HLA-DR is expressed on monocytes and dendritic cells allowing the distinction from basophils and eosinophils. The latter two types of cells have different size and granularity and can thus be distinguished using forward scatter and side scatter characteristics.
[2]CD3 is expressed on T cells and therefore allows the exclusion of this cell type when using CCR3 or CRTH2. The distinction between basophils and eosinophils can be done by size and granularity using forward scatter and side scatter.
[3]CD203c is specific for basophils and therefore can be used to identify basophils without other markers.
Abbreviations: CCR3, C-C chemokine receptor type 3; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells.

In peripheral blood, IgE is detected on basophils, dendritic cells, eosinophils, monocytes, macrophages, B cells and platelets. The expression of IgE on the surface of basophils varies with the atopic status, being higher in atopic individuals. Labelling basophils with an anti-IgE antibody can activate the cells. This undesired effect can be reduced by fixing, cooling and adding EDTA-containing buffer to the cells before staining.

CD123 is the low affinity (α) subunit of the IL-3 receptor. It is expressed in high levels on plasmacytoid dendritic cells and basophils and in low levels on monocytes, eosinophils, myeloid dendritic cells and subsets of haematologic progenitor cells. Additional staining with HLA-DR discriminates between HLA-DR-negative basophils and HLA-DR-positive dendritic cells and monocytes. One of the advantages of identifying basophils with CD123 and HLA-DR is that their expression is not influenced by the allergic status of the donor.

CCR3 is the receptor for C-C type chemokines (e.g. eotaxin, MCP and RANTES). It is highly expressed on basophils and eosinophils but also on Th1 and Th2 cells. Thus, an anti-CD3 marker should be used in combination with CCR3 to exclude the CD3 positive T cells. Side scatter allows distinguishing basophils from eosinophils. Haussmann et al[66] have compared the mentioned three basophil identification methods (IgE, CD123/HLA-DR and CCR3) and showed that CD123/HLA-DR and CCR3 are the most reliable. CCR3 has the advantages of being stable with the atopic background of the patient and of allowing the identification of basophils with a single marker; however, CCR3 has the disadvantage of being down-regulated after basophil activation[66].

CRTH2 is another marker that is expressed by basophils, eosinophils and T cells, and thus requires a T cell marker, such as CD3, to distinguish basophils from T cells. CD203c is constitutively and specifically expressed on basophils and therefore can be used as a single identification marker.

1.7.2 Basophil activation markers

Following stimulation with allergen, the expression of different proteins is up-regulated on the surface of basophils[64], namely CD63[62] and CD203c[67, 68](Table 1.4). CD63 is a lysosomal-associated membrane protein (LAMP), which is not expressed on the surface of resting basophils but only on the membrane of the granules inside the cells[62]. When the granules fuse with the plasmatic membrane of the basophils during degranulation, CD63 becomes expressed on the surface of basophils[68]. CD203c is an enzyme that cleaves phosphodiester and phosphosulphate bonds, hydrolytically removing 5'-nucleotides successively from the 3'-hydroxy-termini of oligonucleotides. It is exclusively and constitutively expressed in low levels on the surface of basophils and mast cells and its expression increases with cell activation.

Table 1.4 Main basophil activation markers.

Abbreviation: LAMP, lysosomal associated membrane protein.

Marker	CD203c	CD63
Synonym	neural cell surface differentiation antigen	gp53, lysosomal associated membrane protein 3 (LAMP-3)
Family	ectonucleotide pyrophospha-tase/phosphodiesterases (ENPP-3)	transmembrane-4 superfamily (tetraspanins)
Property	glycosylated type II transmembrane molecule	secretory granule-associated protein involved in vesicle fusion events
Cells expressing in peripheral blood	exclusively and constitutively expressed on basophils	basophils, neutrophils, eosinophils, monocytes, and platelets
Expression in resting basophils	low expression can also be used as an identification marker	anchored to the intracellular granules and barely expressed on the surface of the membrane
Expression in IgE-activated basophils	levels of CD203c rapidly increase unimodal expression	up-regulated on the surface as a result of fusion between the granule and the plasma membrane bimodal expression
IL-3 priming	sensitive to IL-3 priming	not sensitive to IL-3 priming
Other markers expressed concomitantly	CD13 CD164	CD107a (LAMP-1) CD107b (LAMP-2)

Basophil activation markers seem to form two distinct groups of markers that are up-regulated concomitantly: one including CD63, CD107a and CD107b and another including CD203c, CD13 and CD164[65] (Figure 1.3). These markers behave differently in their up-regulation profiles[69, 70]. The increase in their expression in response to specific activators and inhibitors follows different kinetics and seems to be directed through distinct signal transduction pathways. For example,

CD203c is expressed in resting basophils that have not been primed with IL-3 and increases after activation in the whole basophil population, whereas CD63 is not expressed in resting cells and its up-regulation is bimodal, with only a subgroup of basophils expressing it. The expression of CD203c is higher on the surface of basophils of atopic when compared with non-atopic patients and also in patients with chronic urticaria[71]. This *in vivo* priming reflects ongoing basophil activation. Interestingly, basal expression of CD203c has shown to be increased in patients with uncontrolled asthma and frequent asthma exacerbations[72]. Dose-response curves with different agonists and inhibitors show dissociation between the two activation markers: CD203c is associated with the low-dose events of chemotaxis and CD63 is associated with degranulation[65]. Different studies have suggested that CD63 may reflect anaphylactic degranulation whereas CD203c may reflect piecemeal degranulation. MacGlashan[73] hypothesised that this may be the reason why neither CD203c nor CD63 strictly reflect the exact amount of histamine released. Histamine release measured in the cell supernatant is an average of what occurs in a heterogeneous population of basophils, being a result of the sum between the two pathways of basophil activation. This reflects an advantage of using flow cytometry to study basophil activation compared to measuring histamine release as it gives more complete and detailed information about the behaviour of individual cells following stimulation with allergen.

1.7.3 Practical aspects of the basophil activation test

The laboratory procedure of the BAT consists of three stages: cell stimulation, cell staining and flow cytometry. The primary source of cells is preferentially whole blood, but dextran- or Ficoll-isolated leukocytes can also be used. When collecting the blood for BAT, a syringe or tube containing anticoagulant should be used and the anticoagulant can vary depending on the adopted laboratory protocol. Blood should be processed as soon as possible, after blood collection, but studies have been performed with samples stored at +4ºC up to 24h[74]. A volume of 75 to 250µl of whole blood is used per condition with equal volume of the stimulants. Crude allergen extracts or purified or recombinant allergens may be used for cell stimulation. Different allergen concentrations should be tested, as the sensitivity of the basophils to specific allergen stimulation varies among patients. With regards to the positive controls, anti-IgE or anti-FcεRI are used to test the integrity of the IgE-mediated pathway and formyl-methionyl-leucyl-phenylalanine (fMLP), a chemotactic agent, to test for the ability of basophils to degranulate through an IgE-

independent mechanism. As a negative control, cells are stimulated with stimulation buffer alone. A short incubation with IL-3 may increase the sensitivity of the assay and has been used in some studies[75]. IL-3 increases the expression of CD203c but not CD63; however, it may cause false positive results[76]. If IL-3 is used, a negative control with IL-3 in stimulation buffer should be added to the experimental plan.

1.7.4 Expressing the results of the basophil activation test

The results of BAT can be determined in terms of percentage of basophils expressing the defined activation marker or in terms of mean fluorescence intensity (MFI) by calculating the stimulation index, i.e. the ratio between the MFI of the selected condition and the MFI of the negative control. The former is usually used for CD63 as CD63 is not expressed in resting cells and its expression after activation is bimodal. The latter is usually used for CD203c which is already expressed in resting cells and its increase following allergen stimulation is unimodal (Figure 1.4).

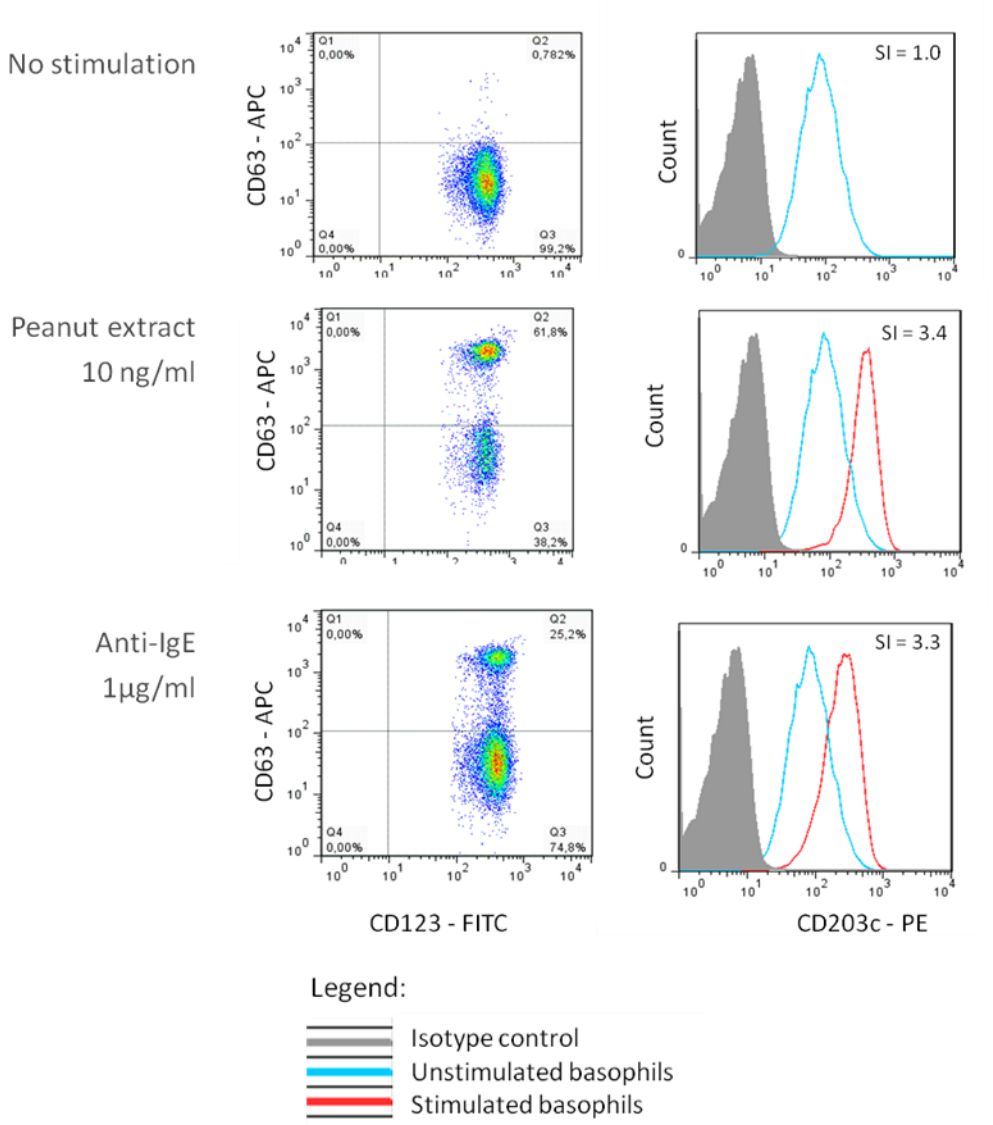


Figure 1.4 Dot plots and histograms showing the expression of CD63 and CD203c on the surface of basophils in different conditions.

Unstimulated cells (negative control) and cells stimulated with peanut or with anti-IgE are represented. The expression of CD63 is represented as the percentage of positive basophils (left panel) and the expression of CD203c is represented as the stimulation index (SI), i.e. the ratio of the mean fluorescence intensity of stimulated cells and the negative control (right panel).

In allergic patients, allergen-induced basophil activation typically results in a bell-shaped dose-response curve, with increasing concentrations of the allergen (usually 5 to 6 log difference) leading to a progressive increase in the expression of the basophil activation markers until reaching a plateau - Figure 1.5. Sub and supra-optimal concentrations can be identified in the dose-response curve. In the example given in Figure 1.5, for the dose-response coloured in blue 100 ng/ml is the optimal concentration of peanut extract and 10 ng/ml and 1000 ng/ml are the sub-optimal and the supra-optimal concentrations, respectively.

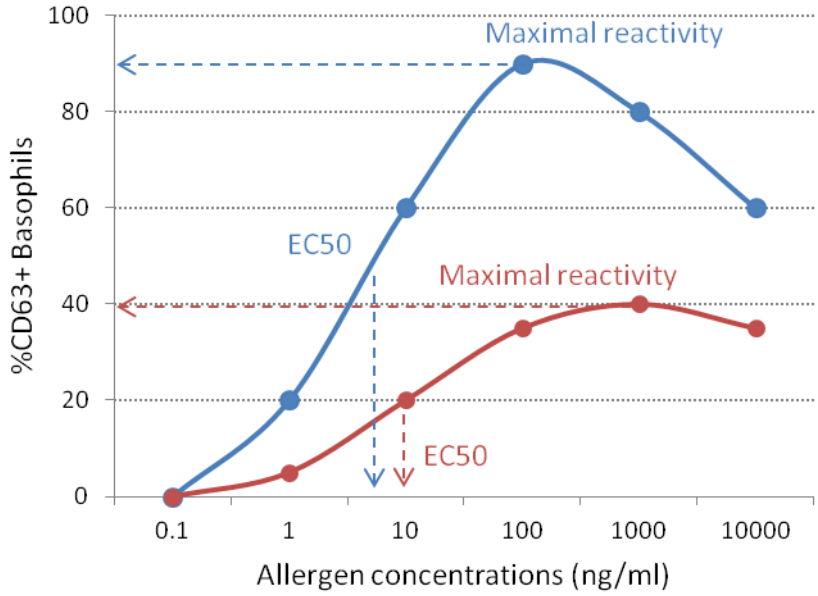


Figure 1.5 Basophil reactivity and basophil sensitivity.

Two examples of dose-response curves of basophil activation following stimulation with various concentrations of allergen are represented. The proportion of CD63+ positive cells is a measure of basophil reactivity and EC_{50}, the effective concentration at 50% of the maximal activation, is a measure of basophil sensitivity.

There is a large degree of variability in the basophil response to allergen between individuals (Figure 1.5). In order to express this heterogeneity and to compare basophil responses between different patients, various parameters can be determined based on the dose-response curve, such as CD-max and EC_{50} or CD_{sens}. CD-max is the maximal activation and corresponds to the maximum proportion of activated basophils at any concentration of allergen. EC_{50} is the effective dose at 50% of the maximal activation, i.e. 50% effective dose, and can also be represented as CD_{sens}. First described by Johansson[77], CD_{sens} is the inverse of the half-maximal effective concentration, i.e. the concentration at which basophil activation is half of the maximum activation, times 100 and can be calculated using the formula: $CD_{sens}=1/EC_{50}x100$.

CD_{max} and CD_{sens} are measures of basophil reactivity and of basophil sensitivity, respectively. Basophil reactivity can be defined as the degree of basophil activation, i.e. the proportion of activated basophils, and can also be measured as the percentage of CD63-positive basophils at different allergen concentrations or as the ratio of the percentage of CD63-positive after stimulation with allergen and with anti-IgE. Basophil sensitivity refers to the concentration of allergen at which basophils become activated and can be expressed as a percentage of the maximal effective dose (e.g. EC_5, EC_{10}) apart from EC_{50} and CD_{sens}, previously mentioned. Figure

1.5 represents the basophil response of two different individuals, one with higher basophil reactivity and sensitivity (blue) and the other with lower basophil reactivity and sensitivity (red), i.e. responding to higher concentrations of the allergen with a smaller proportion of basophils becoming activated. Shreffler and Patil[78] have recently proposed a novel parameter to measure basophil responses, the area under the dose-response curve, which has the advantage of combining basophil reactivity and basophil sensitivity.

1.7.5 Non-responder basophils

In about 5-20% of individuals, basophils do not respond to allergen or to any IgE-mediated stimulants but only to non-IgE-mediated stimulants, such as fMLP - these are the so-called "non-responders". In terms of assessing the individual's response to the allergen for diagnostic purposes, in such patients the result of BAT is uninterpretable. From an immunologic perspective, the non-responder phenotype raises fascinating questions, namely whether this phenomenon could explain the absence of clinical symptoms in sensitised individuals. Although the expression of FcεRI, the amount of IgE bound to FcεRI, the structure of FcεRI and their ability to aggregate following IgE cross-linking seems to be conserved, there is no increase in intracellular calcium and no release of pre-formed or any other type of mediators from non-responder basophils[79]. Defects in Syk, which is present in the early phase of the intracellular signalling pathway leading to basophil degranulation, have been described in these individuals[79-81]. Interestingly, deficiency in Syk does not seem to be present in other types of leukocytes (including B cells, eosinophils, and neutrophils) from the same donors. mRNA levels of Syk are similar between responders and non-responders, suggesting a translational or a post-translational mechanism. Furthermore, this Syk deficiency seems to be reversible following culture of basophils in the presence of IL-3[80]. Unravelling the mechanisms by which non-responder basophils do not become activated following exposure to allergen to which the patient is sensitised to may lead to potential novel treatments for allergic disease.

1.7.6 Assessing basophil intracellular signalling using flow cytometry

The molecular mechanisms governing basophil activation are complex and not completely understood. Traditionally, analysis of cell signalling has been performed using western blot and

ELISA techniques, whose results are an average of the total cell population, which can be heterogeneous[56]. Recently, a proof of concept study demonstrated that flow cytometry can be used to quantify phosphorylation of p38-MAPK in basophils at the same time that the expression of basophil activation markers is evaluated on the surface[82]. Similar methods may be used to evaluate consecutive phosphorylation of other proteins involved in the signalling cascade, similar to what has been done for other cell signalling pathways[83].

Flow cytometry offers advantages over the traditional techniques. It allows identification of cells with heterogeneity in response to activation, it combines surface with intracellular staining and integrates immunophenotyping of individual cells. Flow cytometry enables the study of cells in their natural environment, avoiding basophil purification and potential interference from additional manipulations. Furthermore, flow cytometry significantly shortens the time of analysis from days to hours and reduces the sampling volume considerably, rendering it more accessible for clinical and research applications. This is particularly important in young children, from whom limited volume of blood can be collected.

1.8 Allergic sensitisation and clinical allergy

Sensitisation is the first stage of the development of IgE-mediated food allergy. Clinically, it is demonstrated by a positive skin prick test (SPT) or detectable allergen-specific IgE antibodies in the serum. However, allergic sensitisation does not always lead to clinical allergy. In fact, the majority of individuals with detectable food-specific IgE do not develop any allergic symptoms when consuming the food[8, 43]. In other words, allergen-specific IgE is necessary but not sufficient for the development of immediate-type food allergy.

1.8.1 Phenotypes of allergic sensitisation

There are different phenotypes of food tolerant children. Firstly, there are tolerant children with no IgE to foods (i.e. no allergic sensitisation) and these account for the majority of individuals in the general population. For example, in the Healthnuts study, in a population of 5300 infants, 80% had no IgE antibodies to peanut, sesame, egg or cow's milk and were tolerant to all foods tested[84]. Non-sensitised non-allergic patients have variable levels of IgG and lymphocyte

proliferative responses to the food in question. Some individuals will develop IgE to foods but only a minority of these will develop food allergy. In other words, IgE sensitisation is more common than clinical food allergy[15, 43]. Thus, there are phenotypes of clinically tolerant children who paradoxically show evidence of allergic sensitisation to the food in the absence of clinical reactivity. In this group, five subgroups can be recognised: antenatal sensitisation; stable sensitisation; pre-allergic sensitisation; post-allergic sensitisation; and desensitisation (Table 1.5). It is possible that different phenotypes of allergic sensitisation have different underlying immunological mechanisms and that in some phenotypes different mechanisms coexist.

Table 1.5 Clinical phenotypes of allergic sensitisation.

Clinical phenotypes of allergic sensitisation
• Antenatal sensitisation • Stable sensitisation • Pre-allergic sensitisation • Post-allergic sensitisation • Desensitisation

Allergic sensitisation may develop *in utero* and be present at birth. Studies have shown detection of allergen-specific IgE in cord blood to various antigens. **Antenatal IgE** can be of maternal origin or less commonly of foetal origin[85, 86]. It is unclear whether IgE sensitisation persists through childhood. The clinical relevance of prenatal IgE and a mechanism relating it to the development of allergy are unclear[87].

In the majority of cases, IgE develops post-natally[85, 87, 88]. In the case of **stable sensitisation**, children can develop IgE for many years and never develop symptoms of allergy. This is best described in respiratory allergy and has also been reported in food allergy[89-91]. IgE sensitised patients who never develop allergic symptoms following food allergen exposure during their life-time may have IgE antibodies of different specificity. For example, some clinically irrelevant sensitisation to foods may be secondary to the development of pollen allergy and the cross-reactivity of IgE antibodies. In a recently published study performed in the Isle of Wight[92], the high prevalence of peanut sensitisation at 18 years of age that was not accompanied by a

corresponding increase in peanut allergy was due to the development of grass or tree pollen allergy and sensitisation to the profilin Ara h 5 or the Bet v 1-homologue Ara h 8 (Table 1.1). Other patients may be sensitised to "true food allergens" but IgE binds to a different part of the allergen that is clinically irrelevant as it is not able to induce effector cell activation and clinical symptoms. Furthermore, IgE can be of lower affinity (i.e. strength of antibody binding to the allergen) compared with the IgE antibodies of patients who are sensitised and develop allergic symptoms. An additional explanation for the absence of allergic symptoms in IgE sensitised individuals is the presence of blocking antibodies that could competitively inhibit the function of IgE, such as IgG4[93].

Latent sensitisation has been described where IgE antibodies to foods are present in clinically non-reactive patients who subsequently become allergic to that specific food - this can be called **pre-allergic sensitisation**. A number of studies have evaluated the prospective association between asymptomatic allergen sensitisation and the later development of allergic symptoms in children and adults. For example, in the LISA birth cohort, about 10% of children sensitised to foods at the age of 2 years developed new-onset food allergy at 6 years and early food sensitisation was identified as a strong risk factor for food allergy at the age of 6 (OR= 4.7; 95%CI 2.0-11.2)[91]. In another birth cohort study, early sensitisation to foods was a strong predictor of the later development of allergic disease, including allergic rhinitis and asthma[94].In sensitised patients who become allergic later in life there may be an increase in the levels and a modification of the qualitative features of IgE, namely the development of additional IgE specificities, i.e. the progressive appearance of IgE binding one part of the allergen to IgE binding additional parts of the allergens (so-called "epitope spreading"), and the increase in the affinity of antibody for the allergens, as a result of somatic hypermutation[90].

Food allergy can spontaneously resolve. Depending on the allergen, food allergy can be outgrown in childhood (common in children with cow's milk or egg allergy) [95-99] or tend to persist into adulthood (common in patients with peanut and tree nut allergies) [97, 100]. Although the acquisition of tolerance is usually preceded by a decrease in food-specific IgE levels, IgE can remain detectable after oral tolerance is established - this can be designated **post-allergic sensitisation**. The typical decrease in food-specific IgE levels that accompanies the resolution of food allergies is used in clinic as an indicator of when to perform oral food challenges to assess the acquisition of tolerance[101]. The decrease in food-specific IgE is often accompanied by an

increase in allergen-specific IgG antibodies, in particular of the IgG4 isotype[102, 103].In patients who outgrow their food allergy, possible underlying mechanisms include: reduced T cell proliferation and elimination of reactive T cell clones, a change in the IgE epitope recognition pattern and/or IgE affinity, and the induction of regulatory T cells leading to the production of blocking antibodies such as IgG4 that are able to interfere with IgE and to inhibit allergen-induced activation of basophils and mast cells, reducing cell reactivity. Allergen-specific basophil suppression can also be a consequence of the changes in the characteristics of IgE. Some of these mechanisms have been described in patients who are in the process of outgrowing milk and egg allergy and tolerate extensively heated forms of these foods whilst still reacting to the unprocessed form of milk and egg[104-107].

In patients submitted to allergen-specific immunotherapy, such as subcutaneous immunotherapy to respiratory allergens[108, 109] and oral or sublingual immunotherapy to foods[110-114]a transient increase is followed by a decrease in allergen-specific IgE levels[110, 111] - so called "**desensitisation**". The decrease in allergen-specific IgE is usually accompanied by an increase in IgG4, IgG1 and IgA antibodies. Unfortunately, for the majority of patients desensitisation is transient and only lasts for a few years[115]. The typical clinical outcome of allergen-specific immunotherapy to date has been an increase in the threshold of reactivity rather than the establishment of absolute long-term tolerance. It is debatable whether this should be designated oral tolerance or transient desensitisation. In allergic patients submitted to oral immunotherapy (OIT) to egg or peanut, after cessation of treatment, within weeks or months, clinical improvement is often lost[111, 116, 117]. In contrast, in children with post-allergic sensitisation, who have outgrown their food allergy, it is extremely unusual for the symptoms to recur. The clinical effects of immunotherapy may result from the direct effect of low dose continuous exposure to the allergen on mast cells and basophils (which can result in the activation of inhibitory intracellular signalling molecules such as SHIP followed by progressive loss of activatory intracellular signalling molecules such as Syk and loss of the high affinity IgE receptor FcεRI on the surface of the cells[59]) and/or from the action of blocking antibodies as a result of the induction of regulatory T cells. There is typically no evidence of changes in IgE epitope binding or affinity and some evidence of regulatory T cell changes[117]. In desensitised patients, a good response is usually maintained for as long as the food is consumed and treatment withdrawal is typically followed by a decrease in allergen threshold levels and increased clinical reactivity in the majority of cases[111,

[116, 117]. From an immunological point of view, the reverse of the mechanisms previously described for desensitisation would occur with a progressive restoration of the high affinity IgE receptor FcεRI on the surface of mast cells and basophils and of the activatory component Syk and suppression of SHIP phosphorylation inside these cells and with a progressive loss of the regulatory T cell response, possibly as a consequence of changes in the methylation patterns of transcription factors such as Fox p 3 (the master Treg switch)[117]. These changes overall would result in an increase in effector cell reactivity to the allergen and in the development of allergic symptoms. In true oral tolerance, the absence of clinical (and effector cell) reactivity would remain regardless of whether the food is consumed or not, probably because T cell clone anergy and deletion are irreversible processes.

1.8.2 Discrepancy between allergic sensitisation and clinical allergy to peanut

Clinically, allergic sensitisation to peanut is demonstrated by the presence of allergen-specific IgE, as evidenced by a positive skin prick test to peanut or detectable peanut-specific IgE antibodies in the serum. Allergic sensitisation is far more common than clinical allergy. This is one of the pillars of the practice of the specialty of allergy and clinical immunology and is well documented in various research studies for different types of allergic conditions, including food allergy.

The higher prevalence of allergic sensitisation compared to challenge-proven peanut allergy has been documented across the globe. For example, in the United Kingdom, in the 8-year review of the Manchester Asthma and Allergy Study (MAAS), the prevalence of challenge-proven peanut allergy among peanut-sensitised school-age children was approximately 10% [43]. This number increased to 22.4% if patients with definite peanut allergy that were not challenged were included. In the United States, 10% of children are sensitised to peanut[10], but only 1.4% are clinically allergic to peanut[23]. In Australia, Allen et al[15] recently reported a higher prevalence of peanut allergy in 12-month-old participants in the Healthnuts study at 3% whereas sensitisation to peanut was 3-times higher, i.e. was present in 9% of infants.

1.9 Diagnosis of peanut allergy

The gold-standard for the diagnosis of peanut allergy is oral food challenge[3, 118]. In clinical practice, most centres use open oral food challenges for diagnostic purposes. In the research setting, double-blind-placebo-controlled-food-challenges (DBPCFC) are required to ensure an unbiased outcome[119]. The gold-standard for the diagnosis of food tolerance is an open oral food challenge; thus, a negative DBPCFC should be followed by an open food challenge. However, oral food challenges are time-consuming, labour intensive, expensive and carry the risk of causing an acute allergic reaction, which is potentially severe[25]. Furthermore, with increasing prevalence and awareness about food allergies there has been an increasing number of requests and Allergy services have some difficulty in responding to demand. Moreover, albeit the gold-standard, oral food challenges may result in indeterminate (2-9%), false negative (3%) or false positive (3%) outcomes[120-125]. Therefore, in clinical practice, whenever possible, the diagnosis of peanut allergy is based on the combination of a history of an immediate-type allergic reaction to peanut and *in vivo* or in *vitro* measurement of IgE sensitisation[126]. Some clinics use peanut-specific IgE alone, others use peanut-skin prick test (SPT) alone and some use the combination of these tests. No clear consensus exists as to which is the best approach. The diagnosis of peanut allergy can be particularly difficult in cases where there is no clear history of peanut consumption. With increasing awareness about food allergy and the fact that many families avoid peanut in the first few years of life, peanut-sensitised children with no history of oral exposure to peanut constitute a considerable proportion of patients seen in Allergy clinics. Thus, a test that could accurately diagnose peanut allergy reducing the need for oral food challenge is desirable and would change clinical practice.

Given that the likelihood of clinical peanut allergy increases as the weal diameter on SPT to peanut or serum peanut-specific IgE level increase, diagnostic decision points based on these determinations have been defined to try to improve the utility of SPT and specific IgE to peanut and to reduce the need for oral food challenge[127-131]. Using a mixed population, partly from a specialised outpatients' clinic and partly birth cohort in the United Kingdom, Roberts et al[129] determined that a SPT to peanut ≥8 mm and a serum peanut-specific IgE ≥15 KU_A/l have a 95% positive predictive value for peanut allergy as confirmed by oral food challenge, similar to the cut-offs previously determined in the United States[127, 128]. Cut-offs have been determined in other populations in other parts of the world[130, 131]. These cut-offs vary in different studies with the type

of oral food challenge performed (e.g. open or double-blind placebo controlled food challenge), the oral food challenge protocol (e.g. the criteria to select patients for oral food challenge, consideration of immediate as well as delayed allergic reactions, and subjective symptoms as well as objective signs as a positive oral food challenge, the criteria for stopping the challenge), the study design (e.g. retrospective or prospective) and the statistical analysis performed[127, 128, 130, 131]. Using the same research methodology, these cut-offs may also be influenced by the prevalence of peanut allergy in the studied population and by the age of the child[131, 132]. Furthermore, these cut-off values used alone can misdiagnose a considerable proportion of children with detectable peanut-specific IgE. For example, in the birth-cohort-based Manchester Asthma and Allergy Study (MAAS), only about 55% of peanut allergic patients had peanut-specific IgE levels above 15 KU_A/l[43]. In another UK study in a population partly birth cohort and partly recruited at a specialised Paediatric Allergy clinic, 74% of peanut allergic had peanut-specific IgE levels below the 95% PPV cut-off[129]. Thus, in a large proportion of peanut-sensitised children, peanut-specific IgE levels or SPT results fall below these cut-offs in non-diagnostic values (the so-called immunological grey area[133] - Figure 1.6) and oral food challenges are required to confirm or rule out the diagnosis of peanut allergy[5, 129]. There are also a proportion of patients who are tolerant and have specific IgE levels above the 95% PPV and patients who are allergic and have specific IgE levels below the 95% NPV. Moreover, the levels of peanut-specific IgE do not necessarily reflect clinical allergic reactions and patients may have low peanut-specific IgE levels and develop severe reactions when eating peanut[129].

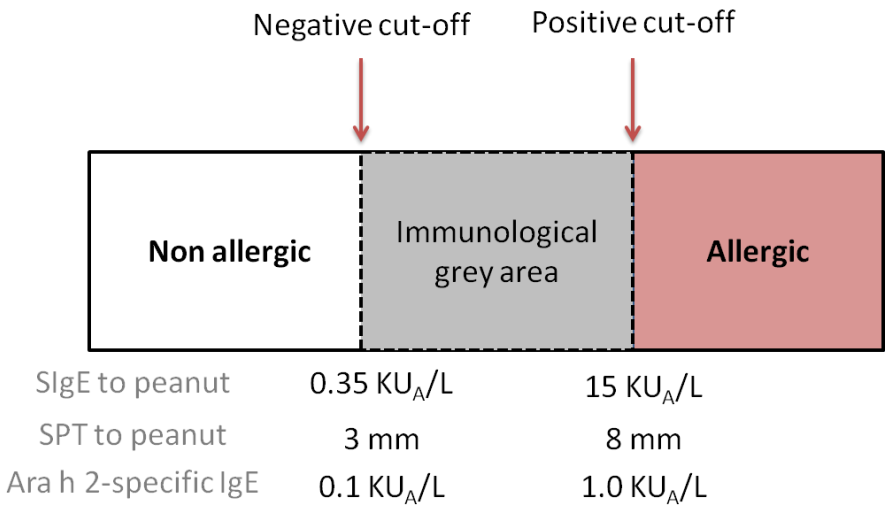


Figure 1.6 Immunological grey area of different allergy tests: specific IgE to peanut, skin prick test and specific IgE to Ara h 2.

Abbreviations: SIgE, specific IgE; SPT, skin prick test.

The determination of specific IgE to peanut components can help distinguishing primary from secondary peanut sensitisation[43, 45, 134]. Ara h 1, Ara h 2 and Ara h 3 are major peanut allergens, usually able to induce systemic reactions in peanut-sensitised patients, whereas Ara h 8 is involved in phenomena of cross-reactivity, causing false positive results on SPT and specific IgE to peanut. Ara h 2 seems to be a dominant allergen and has proved to be particularly useful for diagnosis[44, 134]; however, peanut allergy can develop in patients with undetectable specific IgE to Ara h 2 and the other major peanut allergens[39, 44, 135].

1.10 Management of peanut allergy

There is no curative treatment for peanut allergy. Current recommendations for the management of food allergy and anaphylaxis are based on expert opinion more than evidence-based randomised controlled trials[4, 136, 137]. The mainstay of the management of peanut allergy currently is strict avoidance of peanut-containing foods and the implementation of an emergency plan to treat accidental allergic reactions, which includes adrenaline auto-injectors in the severe cases.

Various clinical factors have been identified as conferring a greater risk for severe food allergic reactions[24], some related to the food (e.g. the allergen involved, peanut being a risk factor for anaphylaxis; the quantity consumed, the type of food processing), some related to the patient (namely the age, with adolescents and young adults being at greatest risk; a previous history of anaphylaxis and the co-existence of uncontrolled asthma) and other related to the circumstances in which the reaction occurs (for example, the absence of cutaneous symptoms, the presence of co-factors such as exercise, alcohol and NSAIDs, delay in administration of adrenaline, concomitant medication that may interfere with treatment such as beta-blockers and angiotensin-converting enzyme inhibitors). However, in studies looking at fatal or near-fatal anaphylaxis, not all patients had such risk factors. For example, the majority who experienced food-related fatal anaphylaxis had previously only had mild allergic reactions[138, 139].

Determination of peanut threshold doses and of whether a patient is likely to react to lowamounts of the allergen is an important aspect of the management of peanut allergy. Individual peanut thresholds could help define the stringency of allergen avoidance measures and population peanut thresholds would be useful for public health authorities and the food industry to establish regulatory measures to protect food allergic patients and to institute allergen control measures

and labelling policies. Thus far, no reliable biomarkers of severity or threshold of acute allergic reactions to peanut or to other food allergens have been identified. The gold-standard to determine the severity of allergic reactions and the threshold dose at which the patients react to is oral food challenge.

1.11 Statement of the problems

More common than developing allergy to peanut is developing peanut-specific IgE, i.e. sensitisation to peanut. In the United Kingdom, 10% of 8-year old children are sensitised to peanut but only 2% have peanut allergy[43]. This means that considering IgE levels alone for the diagnosis of peanut allergy, specific IgE testing will be inaccurate in 4 out of 5 cases. The diagnosis is particularly challenging in the cases where peanut-specific IgE falls in non-diagnostic levels and/or there is no history of oral exposure to peanut or there is a discrepancy between the clinical history and allergy test results, which constitute a considerable proportion of patients seen in Allergy clinics. Moreover, some children have high levels of peanut-specific IgE and tolerate peanut whilst others have low peanut-specific IgE levels and react when exposed to peanut proteins[129].

1.11.1 Diagnosis of peanut allergy

The determination of peanut-specific IgE either by serology or skin prick test carries a significant number of false-positive results. The use of these tests in the clinic requires the capacity to perform oral food challenges to clarify the equivocal cases. The increase in demand has been significant over the recent years. Furthermore, oral food challenges are resource-intensive and carry the risk of causing acute allergic reactions. Thus, a test that could accurately diagnose peanut allergy and reduce the number of oral food challenges could change clinical practice.

1.11.2 Estimating the severity and threshold of allergic reactions to peanut

In the cases where peanut allergy is confirmed, the severity and threshold of allergic reactions is a common concern of patients and their families. The current gold-standard to determine the severity of allergic reactions and the threshold doses at which the patients react to peanut is a

graded double-blind-placebo-controlled-peanut-challenge in patients known to be peanut allergic[140]. This is logistically and technically demanding and carries significant risk; therefore, an *ex vivo* objective biomarker that could reflect the likelihood of experiencing severe allergic reactions for individual patients and estimate threshold levels without the need for DBPCPC would be very valuable to the management of peanut allergy. Determination of thresholds is also useful for the Public Health Authorities and for the Food Industry.

1.11.3 Discrepancy between allergic sensitisation and clinical allergy

Apart from diagnostic difficulties, the discrepancy between allergic sensitisation and clinical allergy to peanut constitutes a gap in our knowledge about the mechanisms of allergy versus tolerance. The presence of IgE is necessary but not sufficient to elicit an acute allergic reaction to peanut. This is an intriguing and still unsolved problem in the field of Allergology that has not been sufficiently explored since the discovery of IgE by Johansson[141] and of its capability of inducing histamine release by Ishizaka and Ishizaka[142].

1.12 Aims

The two main objectives of the present research project were:

1. To develop a new biomarker for peanut allergy, improving the diagnosis of peanut allergy and reducing the need for oral food challenges to peanut;

2. To understand the discrepancy between allergic sensitisation and clinical allergy to peanut.

1.13 Hypotheses

IgE-mediated allergic reactions have been reproduced *in vitro*, where basophil activation markers are analysed by flow cytometry following stimulation with allergen[64, 69, 143]. With respect to the first objective, I hypothesised that the basophil activation test could become a new biomarker for peanut allergy, improving the diagnosis of peanut allergy and reducing the number of oral food challenges. I also hypothesised that the basophil activation test could reflect the severity and threshold of allergic reactions to peanut; and that patients with severe reactions would show

greater basophil reactivity and that patients who react to lower doses of peanut allergen would show greater basophil sensitivity.

With respect to the second objective, I addressed two non-mutually exclusive hypotheses to explain the discrepancy between clinical allergy and allergic sensitisation to peanut:

1. The first hypothesis was that allergen-specific IgE is different in allergic and in sensitised but tolerant patients.

2. The second hypothesis was that sensitised but tolerant patients have an inhibitor that blocks the function of allergen-specific IgE.

Given that natural tolerance to food allergens is allergen-specific and long-lasting, the IgE inhibitor is likely to be a food-specific antibody of an isotype other than IgE, such as IgG4. IgG4 has been shown to increase in patients who naturally outgrow IgE-mediated food allergy, such as cow's milk allergy[102, 103], and in patients who are submitted to food oral immunotherapy[110, 112] and immunotherapy to respiratory allergens[108, 109]. IgG4 is produced as part of a Th2-type immune response, induced mainly by the tolerogenic cytokine IL-10[144], and was therefore the main suspect of being the IgE inhibitor in peanut-sensitised but tolerant patients in this study.

Chapter 2 Material and methods

2.1 Study populations

Within the scope of the present doctoral thesis, three cohorts of patients were studied:

- a population recruited from two specialised Paediatric Allergy outpatients' clinics, the Paediatric Allergy Clinic at St Thomas' Hospital and the Paediatric Allergy Clinic at the Portland Hospital, both in London (BAT Study);
- participants in the Peanut Allergy and Sensitisation study (PAS Study), who are patients excluded from a clinical trial on the prevention of peanut allergy, the Learning Early About Peanut Allergy (LEAP) study; and
- participants in a study of peanut oral immunotherapy at Addenbrooke's Hospital in Cambridge (STOP I Study).

2.1.1 BAT Study

Peanut allergic, peanut sensitised but tolerant and non-sensitised non-allergic children consecutively attending the Paediatric Allergy clinic at St Thomas' Hospital and at the Portland Hospital, in London, were invited to participate in the study. The allergic status to peanut was determined by oral food challenge, except for: (a) children with a convincing history of systemic reaction(s) to peanut within one year of their visit and (i) a wheal size on skin prick test to peanut greater or equal to 8 mm[129] and/or (ii) peanut-specific IgE greater or equal to 15 KU_A/l[129], who were considered peanut allergic; and (b) children who were able to eat 4 grams or more of peanut protein twice a week (as assessed by a validated peanut consumption questionnaire[145]) without developing allergic symptoms, who were considered peanut tolerant. Peanut sensitisation was defined by a weal diameter on skin prick test to peanut greater or equal to 1 mm and/or a serum peanut-specific IgE greater or equal to 0.10 KU_A/l. Children clinically unwell, who had significant chronic illness or were unwilling to participate in the study were excluded.

All children received standard clinical care, including clinical evaluation, skin prick testing (Section 2.2.1), determination of serum specific IgE to peanut and to peanut components (Section 2.3.1.) and oral food challenge (Section 2.2.3.1.), if clinically indicated. A sample of blood for the

research study was collected at the same time and in addition to the blood collected for diagnostic purposes (Section 2.2.2.).

The study was reviewed and approved by the South East London Research Ethics Committee 2 (10/H0802/44) and by the Research & Development Offices of Guy's and St Thomas' Hospital NHS Foundation Trust (RJ110/N300) and of the Portland Hospital (CTO/10/054). Written informed consent was obtained from parents of all children before any study procedures.

2.1.2 PAS Study

The Peanut Allergy and Sensitisation (PAS) study included children that were screened but excluded from the Learning Early About Peanut Allergy (LEAP) study (http://www.leapstudy.co.uk/). The LEAP study is a randomised controlled trial, registered at http://ClinicalTrials.gov with identification number NCT00329784, in which infants at high risk for peanut allergy, as demonstrated by eczema, egg allergy, or both, were enrolled. Participants were stratified based on the results of skin prick test to peanut into those with a weal diameter of 0 mm (SPT-negative stratum or Group II), and those with a weal diameter of 1, 2, 3, or 4 mm (SPT-positive or Group III)[8]. Participants in each stratum were randomly assigned to introduce peanut in the diet or to avoid peanut until 60 months of age. The group assigned to receive a peanut-containing snack or peanut butter ate a minimum of 2 g of peanut protein three times per week. The prevalence of peanut allergy was compared between the peanut consumption and the avoidance groups by oral food challenge at the age of 60 months. At the end of this period, participants were invited to enrol in the LEAP-On study in which participants in both arms of the LEAP study avoid peanut for 12 months. The prevalence of peanut allergy will be re-assessed by oral food challenge at the age of 72 months.

The PAS Study included patients in groups I and IV of the LEAP screening study, i.e. included patients who had to be excluded from the LEAP study either because they did not meet the inclusion criteria (Group I, for example, did not have severe enough eczema) or because they were considered to have already developed peanut allergy as determined by a skin prick test of 5 mm or more in the first year of life (Group IV). These patients were not followed up for the duration of the LEAP study and were invited to participate in the PAS Study at the time of what would have been their last LEAP visit if they had entered the LEAP study. Participants of the PAS

Study underwent the exact same assessments as the LEAP study participants at the end of the LEAP study, namely clinical evaluation, skin prick testing, determination of specific IgE to peanut and to peanut components, oral food challenge and basophil activation test to peanut.

Only data regarding participants in the PAS study, and not data regarding participants in the LEAP and LEAP-On studies, are included in this doctoral thesis.

2.1.3 STOP I Study

The Study of Tolerance to Oral Peanut (STOP I), registered at http://ClinicalTrials.gov with identification number NCT01259804, is an uncontrolled clinical trial of 22 peanut allergic children, aged from 4 to 18 years old, treated with high dose oral immunotherapy (OIT) to peanut[146]. The diagnosis of peanut allergy was established based on a positive oral peanut challenge and the presence of serum peanut-specific IgE. Exclusion criteria were major immunodeficiency and inability to adhere to the OIT protocol. An up-dosing phase with 2 weekly increments to the maximum tolerated dose aiming at 800 mg of peanut protein a day (corresponding to 5 peanuts) over 8 to 38 week-period was followed by a 30 week maintenance phase, where 800mg of peanut protein were taken daily. After 6 and 30 weeks of the maintenance phase, children underwent an open peanut challenge with roasted peanuts.

Dr Andrew Clark, principal investigator of the STOP I study, kindly provided plasma samples from 19 patients included in the study, 14 paired pre and post OIT samples and 5 unpaired post-OIT samples. The post-OIT samples were from the 24-month (5 paired and 3 unpaired samples), 18-month (3 paired samples), 12-month (4 paired and 1 unpaired) or 6-month (2 paired and 1 unpaired) visits.

2.2 Clinical procedures

The clinical procedures for participants in the BAT and in the PAS studies were similar and are described in this section. The clinical assessments of participants in the STOP I study were performed at Addenbroke's Hospital in Cambridge by the research team led by Dr Andrew Clark.

2.2.1 Skin prick testing

Skin prick testing was performed on the volar surface of the non-dominant forearm or on the back of the child using a single-head lancet (ALK-Abelló, Denmark), a positive control (10 mg/ml histamine dihydrochloride, Stallergenes, France), a negative control (50% glycerol and 50% buffered saline, Stallergenes, France) and peanut extract (ALK-Abelló, Denmark). Skin reactions were recorded after 15 minutes. The size of the weal was determined as the mean of two perpendicular diameters including the longest one.

2.2.2 Collection of blood samples

Blood samples were collected simultaneously, i.e. in the same blood draw, for serology, whole blood basophil activation assays and plasma separation for later use in passive sensitisation basophil and mast cell assays. For whole blood basophil activation assays, blood was collected in lithium heparin (BD Vacutainer®). For serology, blood was collected in spray-coated silica tube with a polymer gel (BD Vacutainer® SST™), which was centrifuged; the serum was then collected with a pipette into cryovials and stored at -80°C for later use. For obtaining plasma, blood was collected in citrate-dextrose solution (Sigma-Aldrich, Poole, UK) in a 1 in 10 dilution; the tubes were centrifuged and the plasma was collected with a pipette into cryovials and stored at -80°C for later use.

2.2.3 Oral food challenges

2.2.3.1 Oral food challenge protocol

For the oral food challenges, US high oleic runner variety of peanut were used to produce 12% fat light roast peanut flour by the Golden Peanut Company. Nutritional information was supplied by Golden Peanut Company. When preparing the challenge recipes, muffins and biscuits were weighed individually to ensure consistent size. Protein level was not tested in the final challenge foods. The challenge was blinded using fruit biscuit or chocolate muffin recipe. In terms of the food matrix employed, the fruit biscuit recipe contained plain wheat flour, bicarbonate of soda, rapeseed oil, dark brown sugar, vanilla extract, mashed very ripe bananas, mixed dried fruit, mixed peel and orange juice; and the chocolate muffin contained plain wheat flour, cocoa powder, baking powder, bicarbonate of soda, soft brown sugar, rapeseed oil, prunes in fruit juice pureed,

oatly milk and vanilla extract. For the open challenges, peanut butter, Bamba or whole peanuts (for children 5 years of age or older) were used. Occasionally, if the participant disliked these, other products were used, such as Reese's peanut butter cups, Peanut M & M's or peanut biscuit.

At the start of the challenge, each child was examined (including temperature, blood pressure, respiratory rate, oxygen saturation, auscultation of the chest, peak expiratory flow (if the child was older than 5 years), capillary refill, height and weight) and fitness for challenge was confirmed. Children with active infection, fever, flairs of asthma, eczema or hay fever in the past 2 weeks, had their challenge postponed. Children must had stopped short acting antihistamines for 48 hours, long acting antihistamines for 1 week, leukotriene receptor antagonists for 24 hours, long-acting β Agonists for 48 hours, before the challenge. If children became unwell with the interruption of their medication for this length of time, they also had their challenge postponed."

Prior to the administration of each meal, the child was evaluated for signs of an allergic reaction and vital signs (temperature, pulse, respiratory rate, blood pressure, oxygen saturation, and peak expiratory flow rate) were monitored. The meals were blinded by a computer-generated random code known only to the dietician. A blinded dose may have been repeated if any of the following occurred: abdominal pain, nausea, chest tightness or pain, abnormal oropharyngeal sensation, or unexplained behavioural change.

Double-blind placebo-controlled food challenges consisted of 6 verum doses and 3 placebo doses randomly interspersed with verum doses up to a cumulative dose of 9.35 g of peanut protein (Table 2.1). Children of 1 to 3 years were given one placebo and 5 verum doses up to a cumulative dose of 4.35 g of peanut protein. The last dose was administered in an unblinded fashion. In infants (aged 1 year or less), the oral food challenges were open up to a cumulative dose of 4.35 g of peanut protein. High-risk patients (i.e. patients with suspected peanut allergy, history of life-threatening food-induced anaphylaxis or SPT ≥7 mm) received an additional lower starting active dose of 0.033g of peanut protein. Some older children received an open oral food challenge for logistical reasons. The open challenges followed the same sequence doses of peanut protein as the double-blind-placebo-controlled-food-challenges.

Table 2.1 Doses of peanut protein in the oral food challenge protocol.

Placebo doses were randomly interspersed with verum doses.

DOSES	Peanut protein (g)
1	0.1
2	0.25
3	0.5
4	1.0
5	2.5
6	5.0

2.2.3.2 Criteria for a positive oral food challenge

After each dose, the child was observed for 20 minutes and the examination was repeated. The results of the examinations were recorded in specific tables and the symptoms recorded as free text in the case report form. A system of consensus, where all attending nurses and doctors observed and discussed the symptoms and signs, minimised observer bias. A table with the major and minor criteria for a positive challenge that needed to be ticked at the end of the challenge ensured that the criteria for a positive challenge were met. Oral food challenges were considered negative when all doses were tolerated. If an allergic reaction developed at any stage following a verum dose, the oral food challenge was considered positive (Table 2.2) and the symptoms treated according to local guidelines. If a reaction followed a placebo dose, the patient was brought in for 2-day challenge (one day placebo and one day verum)[147].

Table 2.2 Criteria for positive oral food challenge to peanut.

A positive oral food challenge was defined by the presence of either ≥1 major criteria or ≥2 minor criteria. An indeterminate oral food challenge was defined as one minor criterion. A negative oral food challenge was defined by the absence of major or minor criteria.

Major criteria	Confluent erythematous pruritic rash Wheezing Stridor Dysphonia / Aphonia ≥ 3 urticarial lesions ≥ 1 site of angioedema Hypotension for age not related to vasovagal episode Evidence of severe abdominal pain that persists for ≥3 minutes
Minor criteria	Vomiting Diarrhoea Persistent rubbing of eyes that last ≥3 minutes Persistent rhinorrhoea that lasts ≥3 minutes Persistent scratching that lasts ≥3 minutes

2.2.3.3 Classification of the severity of allergic reactions to peanut

Allergic reactions to peanut on DBPCPC were attributed a symptom score varying between 1 and 5 and the severity was classified into mild (symptom score of 1 or 2), moderate (symptom score of 3) or severe (symptom score of 4 or 5), using published criteria[148]. Patients were grouped depending on whether their reaction was mild/moderate or severe (Table 2.3).

Table 2.3 Classification of the severity of allergic reactions to peanut

Symptom score		Severity classification
1	Localised cutaneous erythema Localised urticaria Localised angioedema Oral pruritus	Mild
2	Generalised erythema Generalised urticaria Generalised angioedema	
3	Gastrointestinal symptoms Rhinitis	Moderate
4	Laryngeal oedema Mild asthma	Severe
5	Dyspnoea Hypotension	

2.2.3.4 Determination of the peanut threshold dose

The threshold dose on oral food challenge was defined as the cumulative threshold dose of peanut protein at the time of reaction as opposed to discrete threshold dose. Patients were grouped according to the cumulative threshold dose at the time of reaction into low (≤0.1g of peanut protein) versus high (>0.1g of peanut protein) threshold. Discrete threshold doses, i.e. the dose administered immediately prior to the positive response, were also recorded[149].

2.3 Laboratory procedures

2.3.1 Measurement of serum immunoglobulins

2.3.1.1 Total IgE and specific IgE to peanut and peanut components

Total and allergen-specific IgE were measured by using an immunoenzymatic assay (ImmunoCAP, Phadia AB, Uppsala, Sweden). Total IgE ImmunoCAP had a measuring range

from 2 to 5000 KU_A/l. Regarding allergen-specific IgE, serum samples were analysed for IgE to peanut as well as IgE to the recombinant peanut allergens Ara h 1, Ara h 2, Ara h 3, Ara h 8 and Ara h 9. The allergen-specific IgE assay had a lower detection limit of 0.01 KU_A/l and an upper detection limit of 100 KU_A/l. For allergen-specific IgE levels above 100 KU_A/l, serial dilutions were performed to determine the exact serum allergen-specific IgE level.

2.3.1.2 Specific IgG4 to peanut and peanut components

Serum specific IgG4 to peanut and to Ara h 1, Ara h 2, Ara h 3, Ara h 8 and Ara h 9 were measured using the Phadia ImmunoCAP 100. The peanut-specific IgG4 assay had a lower detection limit of 70 µg/l and an upper detection limit of 30 mg/l.

2.3.1.3 Specific IgA to peanut

Serum specific IgA to peanut was measured using the Phadia ImmunoCAP 100. The peanut-specific IgA assay had a lower detection limit of 1 mg/l and an upper detection limit of 100 mg/l.

2.3.1.4 Ratios of allergen-specific IgG4 to IgE

The ratios of IgG4 to IgE to peanut and peanut components were determined following conversion of the IgE levels from KU_A/l to ng/ml[150] and conversion of the IgG4 levels from mg/l to ng/ml, using the formula:

$$\text{IgG4/IgE ratio} = (\text{IgG4} \times 1000)/(\text{IgE} \times 2.4)$$

2.3.2 Antibody depletion

2.3.2.1 IgG4 depletion

IgG1 anti-IgG4 antibodies (clone MH164-4, Sanquin, Netherlands) were coupled to CNBr-activated sepharose (GE Healthcare, Hertfordshire, UK) during an overnight incubation at 4°C. Remaining reactive groups were blocked with 1M ethanolamine followed by three cycles of washes in alternating pH using 0.1 M acetic acid/sodium acetate at pH 4.0 and 0.1M Tris-HCl at pH 8.0. Mock-coupled sepharose beads were processed in parallel with anti-IgG4 coupled beads.

Plasma samples were filtered and diluted 1:10 in PBS-AT (0.3% BSA, 0.1% Tween 20 and 0.05% NaN_3 in PBS). Diluted plasma samples were incubated with anti-IgG4- or mocked-coupled sepharose beads in a total volume of 500 µl overnight at room temperature with continuous end-over-end rotation. Anti-IgG4 depleted and mock-depleted samples were collected by means of centrifugation. Total IgG4 plasma levels were measured by ELISA in IgG4-depleted and mock-depleted samples, as described below in section 2.3.2.2.[151]. In the same samples, peanut-specific IgG4 levels were determined using ImmunoCAP, as described above in section 2.3.1.2.

2.3.2.2 IgG4 ELISA

ELISA plates (Nunc Immunoplate Maxisorp, Thermoscientific) were coated with 100 µl per well of purified mouse anti-human IgG4 antibody (clone JDC-14, BD Biosciences) diluted in carbonate buffer (4 nM, pH 9.2) to 2 µg/ml and incubated overnight at 4°C. Following removal of the coating antibody, plates were blocked with 200 µl per well of 1% BSA/PBS and incubated for 1 hour at room temperature. Plates were then washed four times with 0.05% Tween 20 in PBS using a squeezy bottle and 50 µl of serum samples or controls were incubated for 16 hours at 4°C. Following four washing steps, plates were incubated with 100 µl per well of 1 µg/ml biotin-conjugated anti-human IgG4 (clone G17-4, BD Biosciences), followed by washing steps and incubation for 30 minutes at room temperature with 100 µl per well of 5 µl/ml streptavidin–horseradish peroxidase (R&D Systems, Minneapolis, Minn). The plates were washed and 50 µl of the substrate solution, 3,3'-5,5'-tetramethylbenzidine (TMB), was added to each well. The plates were incubated in the dark for 15 minutes and the reaction was stopped with 50 µl per well of 3M sulphuric acid. IgG4 was detected with TMB (R&D Systems) by measuring absorbance at 450 nm. Human IgG4 (Sigma-Aldrich) was used to generate a standard curve from which the concentrations of test samples were extrapolated.

2.3.2.3 IgG depletion

In the collaborative project described in Section 6.2. (entitled "Auto-anti-IgE antibodies"), to deplete total IgG from the sera, 500 µL of serum samples were incubated with an equal volume of protein G Sepharose (Sigma-Aldrich) at 48°C overnight in a mini Bio-Spin chromatography column (Bio-Rad, Hercules, Calif). The flow-through was recovered and re-incubated with fresh

protein G Sepharose at 4°C overnight. IgG was measured in the final recovered flow-through by ELISA, as described in Section 2.3.2.4.

For the affinity experiments described in section 2.3.4., to deplete IgG from plasma, protein G spin columns (GE healthcare, 28-9031-34) were used following the manufacturer's instructions.

2.3.2.4 IgG ELISA

To assess the efficiency of the IgG depletion, total IgG was measured by ELISA. Nunc Maxisorp plates were coated with 100 µl per well of goat anti-human IgG (Oxford Biotech 204001) in carbonate buffer (110 ml Na2CO3 (0.2 M), 140 ml NaHCO3 (0.2 M), 500 ml distilled H2O, pH 9.2) and incubated overnight at 4°C. Following four consecutive washes with PBS/0.05% Tween, plates were blocked with 200 µL per well PBS/Tween/2% marvel for 1 hour at room temperature on a plate shaker. After another four consecutive washes with PBS/0.05% Tween, 50 µL per well of test samples, IgG standard curve (Sigma I4506 in NaCl) or buffer alone (blank) were added to different wells in duplicate, diluted 1:2 from 200 ng/ml in PBS/Tween/1% marvel. The plates were incubated overnight at 4°C. Following another four washing steps, 100 µL of anti-IgG-HRP (Sigma A0170) in PBS/Tween/1% marvel were added per well and the plates were incubated for 2 hours at 37°C. After another four washing steps, 50 µl per well of TMB substrate was added and the reaction was stopped with 50 µL of 1 M sulphuric acid. IgG was detected by measuring absorbance at 450 nm.

2.3.3 Depletion and purification of IgE-binding proteins

In the collaborative project described in Section 6.2. (entitled "Auto-anti-IgE antibodies"), to deplete IgE-binding proteins, IgE was cross-linked to cyanogen bromide (CNBr)–activated Sepharose 4B (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 100 mg of recombinant IgE anti-NP-BSA (manufactured in-house) were mixed with 100 ml of 1 mM HCl-swollen CNBr-activated Sepharose in coupling buffer (0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl) at 4°C overnight in a mini Bio-Spin chromatography column, then washed with coupling buffer. Unconjugated sites were blocked with 0.2 M glycine buffer pH 8.0 for 2 hours at room temperature. After extensive washing with 5 cycles of the coupling buffer and 0.1 M acetate buffer pH 4, the IgE-coupled Sepharose was ready to be used. IgE-coupled Sepharose (50 ml) was

mixed with 3 ml of subjects' sera at 4°C overnight. The eluate containing non-IgE binding antibodies was stored at -20°C for later analysis. Proteins bound to the IgE-coupled Sepharose were then eluted with 100 ml of 0.2 M glycine pH 2.5 into an equal volume of 100 mM Tris buffer pH 8.0. The eluted solutions were dialyzed in PBS at 4°C overnight, and then mixed with protein G Sepharose one-to-one at 4°C overnight to capture IgG antibodies. The IgG anti-IgE antibodies were eluted off the protein G Sepharose with glycine, dialyzed with PBS as above, then quantified by ELISA and run on 10% SDS-PAGE under non-reducing conditions compared with recombinant IgE and IgG (Sigma-Aldrich). No contaminating IgE was found in purified antibodies.

2.3.4 Peanut extract and purified peanut allergens

The peanut extract (Batch EC-B044) used in the basophil activation assay was prepared at ALK Abelló, in Horsholm, Denmark, and kindly provided by Dr Henning Løwenstein. Raw peanut was extracted in cold isotonic phosphate buffer pH 6.5 at 100 mg/ml and magnetically stirred for 90 ± 15 minutes at 2-8°C. The extract was centrifuged at 20,000xg for 30 ± 5 minutes at 2-8 °C then clarified by filtration through glass-fibre filters or 0.5 μm membrane filters. The pH was adjusted to 6.5 ± 0.5. The extract was sterilised by filtering it twice through 0.2 μm filters then filled at 1 ml per vial into sterilised glass vials and freeze dried. The frozen lyophilised extract was gradually brought to room temperature and reconstituted with 1 ml of PBS at 20 mg of protein per millilitre before use. The extract concentrations are expressed as mass of protein per unit of volume throughout the thesis.

The concentration of protein in the peanut extract was determined at ALK-Abelló as being 20 mg/ml. Stability studies were performed where chemical, biochemical and microbiological parameters were tested after 24 months of storage at 2-8°C, after 6 months of storage at 25°C, after 15 days of storage at 37°C and after 6 months of storage at 2-8°C, under in-use conditions. In all these conditions, the extract showed to be stable and maintained its protein profile as assessed by SDS-PAGE electrophoresis and its allergenic activity as assessed by RAST inhibition with an in-house reference.

The presence of Ara h 1, Ara h 2 and Ara h 3 in the peanut extract was confirmed by western blotting. The concentration of the major peanut allergens Ara h 1 (5.5 mg/ml), Ara h 2 (1.6 mg/ml) and Ara h 3 (4 mg/ml) in the crude peanut extract was measured by SDS-PAGE and densitometry

(Figure 2.1) by Dr Soheila Maleki (Agriculture Research service, Southern Region Research Center, New Orleans, Louisiana, USA).

Dr Maleki also kindly provided purified native peanut proteins, namely Ara h 1, Ara h 2, Ara h 3 and Ara h 6[152, 153].

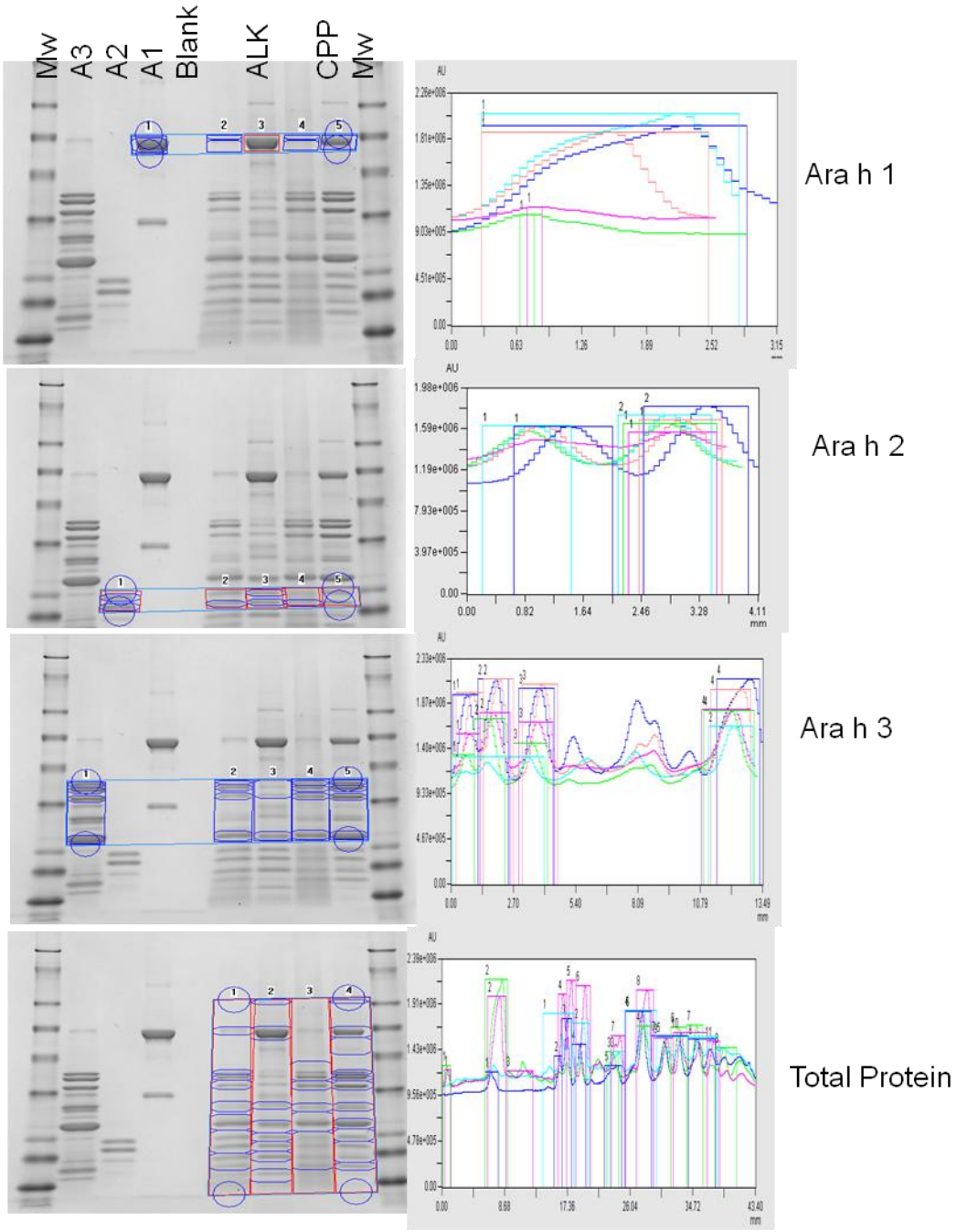


Figure 2.1 Quantification of Ara h 1, Ara h 2 and Ara h 3 using SDS-PAGE and densitometry.

Abbreviations: A3, purified Ara h 3; A2, purified Ara h 2; A1, purified Ara h 1; ALK, peanut extract from ALK-Abelló; CPP, crude peanut protein; MW, molecular weight marker. Courtesy of Dr Soheila Maleki.

2.3.5 Surface plasmon resonance

Surface Plasmon resonance experiments were performed using a Biacore T200 instrument. All experiments were performed at 25°C. A fusion protein mimicking the high affinity IgE receptor formed by the soluble fragment of the high-affinity IgE receptor α-chain fused to the Fc region of IgG4[154], designated IgG4-Fc(sFcεRIα)$_2$, kindly provided by Dr Tihomir Dodev and Dr Andrew Beavil, was used to capture IgE. Purified native Ara h 2 was injected over the sensor chip containing IgE bound to the fusion protein to measure IgE affinity for the peanut allergen.

The aim was to compare the IgE affinity for peanut allergen Ara h 2 between peanut allergic and peanut sensitised but tolerant patients. However, peanut sensitised but tolerant patients tended to have lower levels of IgE to peanut and to peanut allergens and lower proportion of IgE that was allergen-specific. Furthermore, most of the peanut tolerant patients with IgE to the major peanut allergens were infants and young children and the volume of plasma samples collected from these study participants was limited. To assess the feasibility of the experiments and the experimental design, pilot experiments using recombinant grass pollen allergen Phl p 7 and plasma from a non-atopic patient spiked with recombinant monoclonal IgE antibody directed to Phl p 7 were performed before testing samples of PA and PS patients.

2.3.5.1 Immobilisation of IgG4-Fc(sFcεRIα)2 fusion protein to the sensor chip

The IgG4-Fc(sFcεRIα)$_2$ fusion protein was diluted to 10 μg/ml in sodium acetate buffer (pH 4.5) and immobilised on the surface of a CM5 chip flow cell (Biacore, Uppsala, Sweden) by amine coupling: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were injected to activate the flow cell followed by injection of IgG4-Fc(sFcεRIα)$_2$ fusion protein. Following this, the flow cell was deactivated with 1 M ethanolamine-HCl (pH=8.5) to prevent further non-specific binding. A second control flow cell was prepared in parallel in which the surface was activated and deactivated in the absence of IgG4-Fc(sFcεRIα)$_2$ fusion protein. IgE-containing plasma and allergen were flown over both a blank surface with no protein immobilised (flow cell 3) and a surface where IgG4-Fc(sFcεRIα)$_2$ was immobilised (flow cell 4).

2.3.5.2 Pilot experiments using monoclonal anti-Phl p 7 IgE and recombinant Phl p 7

Pilot experiments using a single recombinant grass pollen allergen Phl p 7 (kindly provided by Dr Louisa James and Professor Hannah Gould) and plasma from a non-atopic patient spiked with recombinant monoclonal IgE antibody anti-Phl p 7 (also provided by Dr Louisa James and Professor Hannah Gould) were performed to test the experimental design before testing samples of PA and PS patients. The aims were to test firstly whether it was possible to capture IgE from plasma using the IgG4-Fc(sFcεRIα)$_2$ fusion chip and secondly whether it was possible to measure allergen-IgE interactions in samples with a low proportion of allergen-specific IgE using a small volume of plasma.

Plasma from a non-atopic adult volunteer was spiked with a mixture of recombinant monoclonal IgE antibody specific for the grass pollen allergen Phl p 7 and polyclonal non-specific IgE (WHO reference standard). The concentration of allergen-specific IgE was adjusted to 100 μg/ml and mixed with non-specific IgE in the following proportions: 100%, 30%, 10%, 3%, 1%, 0.3% and 0.1%. A plasma sample from a non-atopic adult volunteer was applied to a protein G spin column (GE healthcare, 28-9031-34) to deplete IgG. The IgE mixtures were added to the non-atopic plasma at 2.5 μg/ml.

The IgE-spiked plasma samples were injected in series over the IgG4-Fc(sFcεRIα)$_2$ and control flow cell surfaces for 600 s at a flow rate of 5 μL/min. Binding of IgE to the IgG4-Fc(sFcεRIα)$_2$ protein was detected as indicated in Figure 2.2. After a 120s stabilization period, recombinant Phl p 7 (1 μM) was injected for 300 s and binding of IgE and of allergen was detected as indicated in Figure 2.2. Between cycles the surface of IgG4-Fc(sFcεRIα)$_2$ was regenerated with glycine (pH 2.5). Binding of allergen was detected when specific IgE was present at 100%, 30% and 10% of total IgE (Figure 2.2.B.).

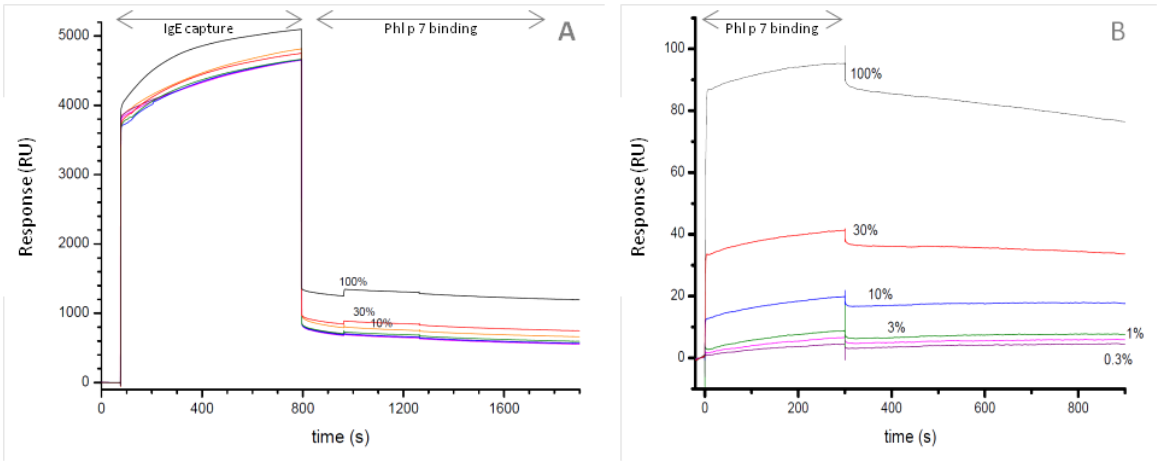


Figure 2.2 Surface plasmon resonance following capture of plasma IgE (including Phl p 7-specific IgE) by IgG4-Fc(sFcεRIα)2 and subsequent flow of recombinant allergen Phl p 7.

A. IgE capture and allergen binding expressed in response units and not corrected for background. B. Allergen binding shown as response units for flow cell 4 (surface where IgG4-Fc(sFcεRIα)2 was immobilised), with background control data from flow cell 3 (blank surface with no protein immobilised) subtracted.

2.3.5.3 Experiments using plasma of peanut allergic and peanut sensitised but tolerant patients and purified Ara h 2

Using the same IgG4-Fc(sFcεRIα)$_2$ immobilised sensor chip as described in Section 2.3.4.2, plasma samples of PA and PS patients (Table 2.4), previously depleted of IgG using protein G spin columns (GE healthcare, 28-9031-34) according to the manufacturer's instructions, were injected for 600s at 5 μL/minute to capture IgE. Purified peanut protein Ara h 2 was then injected for 300s at 5 μl/minute at increasing concentration (1nm to 1μM) to detect binding to Ara h 2-specific IgE captured on the sensor chip. The kinetics of binding was analysed using the BIAevaluation software. Between cycles the surface of the sensor chip was regenerated with glycine, pH 2.5.

Unidentified proteins specifically present within plasma from atopic patients bound to both the IgG4-Fc(sFcεRIα)$_2$ and to the control surface leading to a high background that was not completely removed by glycine regeneration. Each injection of plasma resulted in an increase in the baseline response until the sensor surface became saturated. Under these conditions it was not possible to detect binding of allergens to IgE. Alternative regeneration conditions (high pH) and the addition of high salt concentrations to the samples were attempted to prevent the observed non-specific binding of plasma proteins to the IgG4-Fc(sFcεRIα)$_2$ surface without success. In light of these technical challenges, the planned affinity measurements of IgE present in the plasma for purified peanut allergens were not achieved.

Table 2.4 Examples of plasma samples of peanut allergic and peanut sensitised but tolerant patients selected to measure affinity of IgE for Ara h 2 using surface plasmon resonance.

The samples were selected on the basis of Ara h 2 ≥0.10 KU/L and ratio of Ara h 2-specific IgE to total IgE ≥0.3% and paired between peanut allergic and peanut sensitised but tolerant for the ratio of Ara h 2-specific IgE to total IgE. Abbreviations: ID, identification; SPT, skin prick test.

Patient group	Patient ID	SPT (mm)	Total IgE (KU/l)	Peanut-specific IgE (KU_A/l)	Ara h 2-specific IgE (KU_A/l)	Ratio of Ara h 2-specific IgE to total IgE (%)
Peanut allergic	AS057	16	483	5.21	5.27	1.09%
	AS040	8	42	0.46	0.34	0.81%
	AS163	10	105	21.6	4.42	4.21%
	PAS8301	10	250	5.81	5.41	2.16%
	PAS8726	8	33	2.45	0.38	1.15%
	AS012	7	40	0.39	0.68	1.70%
	AS067	8	324	7.01	1.24	0.38%
Peanut sensitised but tolerant patients	AS079	10	8132	97.1	82.30	1.01%
	AS120	2	13	0.05	0.11	0.85%
	AS136	4	6	0.56	0.24	4.00%
	AS140	2	45	0.14	0.17	0.38%
	AS151	5	13	0.71	0.28	2.15%
	AS175	7	305	0.41	0.12	1.20%
	AS182	7	7	0.07	0.13	1.86%

2.3.6 Basophil assays

2.3.6.1 Whole blood basophil activation assay

2.3.6.1.1 Laboratory procedure

Peripheral venous blood was collected in lithium heparin tubes (BD Vacutainer®, Plymouth, UK) and tested within four hours of collection. Whole blood (100 µl) was stimulated for 30 minutes at 37˚C with an equal volume of peanut extract (ALK Abelló, Horsholm, Denmark) diluted in Rosewell Park Memorial Institute medium (RPMI, GIBCO, Paisley, UK) at serial 10-fold dilutions

from 10 µg/ml to 0.1 ng/ml. Polyclonal goat anti-human IgE (1 µg/ml, Sigma-Aldrich, Poole, UK), monoclonal mouse anti-human FcεRI (2.5 µg/ml, eBioscience, San Diego, CA, USA), formyl-methionyl-leucyl-phenylalanine (fMLP, 1 µM, Sigma-Aldrich, Poole, UK) or RPMI alone were used as controls. The reaction was stopped by adding cold ethylenediaminetetraacetic acid (EDTA) at a final concentration of 20 mM. Cells were stained for 30 minutes at 4˚C with the following fluorochrome-conjugated monoclonal antibodies: CD123-fluorescein isothiocyanate (clone 6H6, eBioscience, San Diego, CA, USA), CD203c-phycoerythrin (clone NP4D6, Biolegend, San Diego, CA, USA), HLA-DR-peridinin chlorophyll protein (clone L243, Biolegend, San Diego, CA, USA) and CD63-allophycocyanin (clone MEM259, Biolegend, San Diego, CA, USA). Red blood cells were lysed with BD Pharmlyse lysing buffer (BD Immunocytometry Systems, San Jose, CA, USA). Basophils were gated as side scatter $(SSC)^{low}$/CD203c+/CD123+/HLADR- unless otherwise indicated, and surface expression of CD63 and CD203c was analysed using FACS Canto II with FACSDiva software (BD Biosciences, San Jose, CA, USA). A minimum of 1000 SSC^{low} CD203c+ CD123+ HLADR- events were acquired.

2.3.6.1.2 Data analyses

BAT data was analysed using FlowJo software version 7.6.1. (TreeStar, Ashland, Ore, USA). Basophil activation was expressed as the percentage of CD63-positive basophils with the background activation (i.e. the percentage of CD63-positive basophils in the negative control) subtracted. Basophil activation was also expressed as the stimulation index of CD203c (SI CD203c), i.e. the ratio of the mean fluorescence intensity of CD203c-PE for each condition and the mean fluorescence intensity of CD203c-PE for the negative control. Other parameters were constructed based on the percentage of CD63-positive basophils or the SI CD203c, namely:

- Mean CD63 Peanut 10-100 - average of the percentage of CD63-positive basophils at 10 and 100 ng/ml of peanut extract;
- Mean CD203c 10-100 - average of the stimulation index of CD203 on basophils at 10 and 100 ng/ml of peanut extract;
- Mean CD63 Peanut 100-1000 - average of the percentage of CD63-positive basophils at 100 and 1000 ng/ml of peanut extract;
- Mean CD203c 100-1000 - average of the stimulation index of CD203 on basophils at 100 and 1000 ng/ml of peanut extract;

- AUC CD63 - area under the dose-response curve for the basophil activation expressed as percentage of CD63-positive basophils using a logarithmic scale for the peanut extract concentrations;
- AUC CD203c - area under the dose-response curve for the basophil activation expressed as stimulation index of CD203c on basophils using a logarithmic scale for the peanut extract concentrations;
- Maximal %CD63 Peanut or CDmax - the maximal percentage of CD63-positive basophils, i.e. the percentage of CD63-positive basophils at the optimal peanut extract concentration;
- Maximal SI CD203c Peanut - the maximal stimulation index of CD203c on basophils, i.e. the stimulation index of CD203c on basophils at the optimal peanut extract concentration;
- %CD63+ Peanut 100/aIgE - the ratio of the percentage of CD63-positive basophils following stimulation with 100 ng/ml of peanut extract and the percentage of CD63-positive basophils following stimulation with anti-IgE;
- %CD63+ Peanut 100/aFcεRI - the ratio of the percentage of CD63-positive basophils following stimulation with 100 ng/ml of peanut extract and the percentage of CD63-positive basophils following stimulation with aFcεRI;
- EC_{50} CD63 - half-maximal effective concentration determined using the CD63 dose-response curve;
- EC_{50} CD203c - half-maximal effective concentration determined using the CD203c dose-response curve;
- CD_{sens} CD63 - the inverse of EC_{50} times 100 determined using the CD63 dose-response curve;
- CD_{sens} CD203c - the inverse of EC_{50} times 100 determined using the CD203c dose-response curve.

2.3.6.2 Basophil activation test to determine biological activity of peanut-containing dust samples

Six extracted dust samples containing high (19.3-43.5 µg/ml, n=3) or low (0.01-0.015 µg/ml, n=3) levels of peanut protein were used to stimulate basophils of 3 peanut monoallergic and 3 non-allergic children, resulting in 5 pairs of dust sample–patient experiments. The extracted dust

samples with high peanut content were diluted to a maximum concentration of 10 µg/ml and then serially diluted to compare dose-responses obtained with the extracted dust samples and the peanut standard across experiments. The low peanut dust samples were prepared using the same dilution factor to control for other potential components of the dust extract that could activate basophils independently of peanut protein. In this group of patients, peanut allergy was diagnosed based on a combination of a recent history of an immediate allergic reaction to peanut and peanut skin prick test responses of 8 mm or greater (Stallergenes, Antony, France) and serum peanut-specific IgE levels of 15 KU_A/l or greater (ImmunoCAP; ThermoFisher Scientific, Uppsala, Sweden)[129]. Non allergic patients were eating peanuts regularly and had negative skin prick test results and negative serum specific IgE levels to peanut and to common food and airborne allergens. Apart from skin prick testing to peanut and to common food and airborne allergens and from specific IgE to peanut, patients were also tested for specific IgE to peanut components Ara h 1, Ara h 2, Ara h 3, Ara h 8 and Ara h 9. Whole blood basophil activation test using the extracted dust samples, the peanut extract and the appropriate negative (RPMI alone) and positive (anti-IgE and fMLP) controls, as described in section 2.3.6.1.

2.3.6.3 Basophil activation test to determine the ability of serum from asthmatics to activate basophils

Citrate-dextrose anticoagulated blood was obtained from a single atopic non-asthmatic donor with total serum IgE >150 KU_A/l and sensitised to house dust mite as determined by skin prick testing. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation using Histopaque-1077 (Sigma-Aldrich, Poole, UK) and resuspended to a final concentration of 2 x 10^7 cells/ml. For each condition, 1 x 10^6 cells (50 µL) were incubated with 50 µL of test sera diluted 1:2 with BAT buffer (HBSS with 2 mM $CaCl_2$) for 30 minutes at 37°C and degranulation stopped by adding 2 ml of FACS buffer containing 2 mM EDTA on ice. Autologous serum or BAT buffer were used as negative controls. Polyclonal goat anti-human IgE, monoclonal mouse anti-human FcεRI or fMLP were used as positive controls. PBMC were stained with anti-CD203c-phycoerythrin (Clone NP4D6; BioLegend, San Diego, Calif) and anti-CD63-allophycocyanin (Clone MEM-259; BioLegend) and analysed by flow cytometry, as previously described. Basophils were gated as SSC^{low} CD203c+ cells and basophil activation was defined as the

percentage basophils expressing the activation marker CD63 compared with the BAT buffer negative control.

2.3.6.4 Basophil intracellular signalling

To assess intracellular signalling during IgE-mediated basophil activation, BD Phosflow™(BD Biosciences, San Jose, California) reagents were used. Blood collection and preparation of stimulants were performed as described in section 2.3.6.1. Whole blood and stimulants were incubated with the following surface-staining antibodies for 30 minutes (unless indicated otherwise) at 37°C (5% CO2) in an incubator: CD123-allophycocyanin (clone 7G3, BD Biosciences, San Jose, CA, USA), HLA-DR-peridinin chlorophyll protein (clone L243, Biolegend, San Diego, CA, USA) and CD63-phycoerythrin-Cy7 (clone H5C6, Biolegend, San Diego, CA, USA). 20 µl of cold (4°C) 20 mM EDTA (in PBS) was added to each tube to stop degranulation. Cells were fixed and lysed with pre-warmed BD Lyse/Fix Buffer and incubated at 37°C for 10 min. Following washing, cells were permeabilised with BD Perm/Wash Buffer I. Cells were washed and resuspended in Perm/Wash Buffer I and stained with BD Phosflow antibodies: Syk-Alexa Fluor 488 or phycoerythrin (clone I120-722 (pY348), BD Biosciences, San Jose, CA, USA), SHP-2-phycoerythrin (clone L99-921 (pY542), BD Biosciences, San Jose, CA, USA) and p38-MAPK-pacific blue or phycoerythrin (clone 36/p38 (pT180/pY182), BD Biosciences, San Jose, CA, USA), depending on the colour panel used. Cells were incubated at room temperature for 60 minutes protected from light, washed and resuspended in BD Perm/Wash Buffer I prior to flow cytometric analysis.

2.3.6.5 Passive sensitisation basophil activation and inhibition assays

Peripheral blood mononuclear cells (PBMC) were isolated from citrate-dextrose anticoagulated blood of atopic non-peanut allergic non-peanut sensitised adult volunteers by density gradient separation using Histopaque-1077 (Sigma-Aldrich, Poole, UK). For stripping of membrane-bound IgE, PBMC were resuspended in lactic acid (13.4 mmol/l lactate, 140 mmol/l NaCl, 5 mmol/l KCl, pH 3.9) and incubated at 4˚C for 5 minutes[155]. Human serum albumin (HSA) 0.5% in RPMI was added and the solution neutralised with 12% Tris. After washing with 0.5% HSA, the cell pellet was resuspended in 1:100 of the original blood volume in 0.5% HSA. The cell suspension was

incubated for 60 minutes at 37˚C with the same volume of individual plasma from study participants. After washing, resensitised PBMC were stimulated with peanut extract or purified peanut proteins, stained and analysed by flow cytometry, as described in section 2.3.6.1.

For inhibition experiments, 20 µL of plasma from a reference PA patient was incubated with 20 µL of plasma from PS or NA patients and 10 µL of allergen (concentration as indicated) before the addition of $1x10^6$ (50 µL) IgE-stripped PBMC to the allergen-plasma mixture. The reference PA plasma contained 172 KU_A/l of specific IgE to peanut, 74.1 KU_A/l of specific IgE to Ara h 1, 77.6 KU_A/l of specific IgE to Ara h 2 and 33.6 KU_A/l of specific IgE to Ara h 3. The same reference PA plasma was used in all basophil inhibition experiments. The results were expressed as percentage of inhibition and calculated using the formula:

% inhibition = (%CD63+ of cells sensitised with PA plasma-%CD63+ of cells sensitised with PA plasma in presence of test plasma)/%CD63+ of cells sensitised with PA plasma.

2.3.6.6 Optimisation of basophil assays

The described basophil assays were developed specifically for this research project.

2.3.6.6.1 Anti-IgE dose response

PBMC were isolated from citrate-dextrose anticoagulated blood of atopic adult volunteers by density gradient separation using Histopaque-1077 (Sigma-Aldrich, Poole, UK) and stimulated with 0.5% HSA in RPMI, 1 µM fMLP or anti-IgE serially diluted in 0.5% HSA RPMI at different concentrations ranging from 0.01 to 100 µg/ml to identify the optimal anti-IgE concentration to be used in future experiments.

Expression of CD63 and CD203c on the surface of basophils followed a bell-shaped dose-response curve (Figure 2.3).

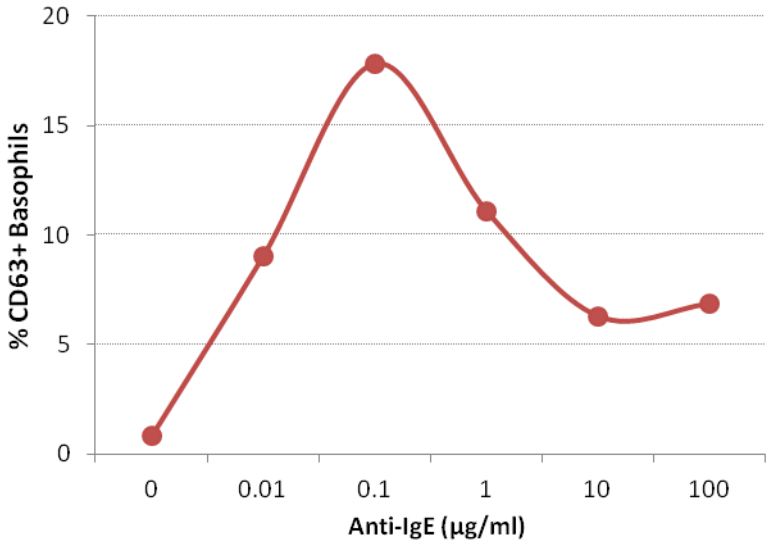


Figure 2.3 Dose-response curve of CD63 expression on the surface of basophils following stimulation with different concentrations of goat polyclonal anti-human IgE.

The optimal concentration of anti-IgE was 0.1 µg/ml or 1 µg/ml in replicate experiments (n=2). Taking into account the variability in basophil reactivity between donors and the reported optimal concentration of anti-IgE (1 to 100µg/ml[69]), the concentration of anti-IgE of 1 µg/ml was adopted for all subsequent experiments unless otherwise indicated.

2.3.6.6.2 Allergen dose-response

Following passive sensitisation of basophils with plasma from peanut allergic patients, stimulation with different concentrations of peanut extract ranging from 0.001 to 10,000 ng/ml was performed. The expression of CD63 and CD203c was detected after stimulation with increasing concentrations of peanut extract, resulting in a bell-shaped dose-response curve. Figure 2.4 illustrates a representative experiment where basophils were sensitised with plasma from a peanut allergic patient with high specific IgE to peanut (172 KU_A/l) and to peanut major allergens, Ara h 1 (74.1 KU_A/l), Ara h 2 (77.6 KU_A/l) and Ara h 3 (33.6 KU_A/l). Prior to passive sensitisation, native and IgE-stripped basophils from the basophil donor did not respond to peanut extract but only to the positive controls, 1 µg/ml anti-IgE and 1 µM fMLP.

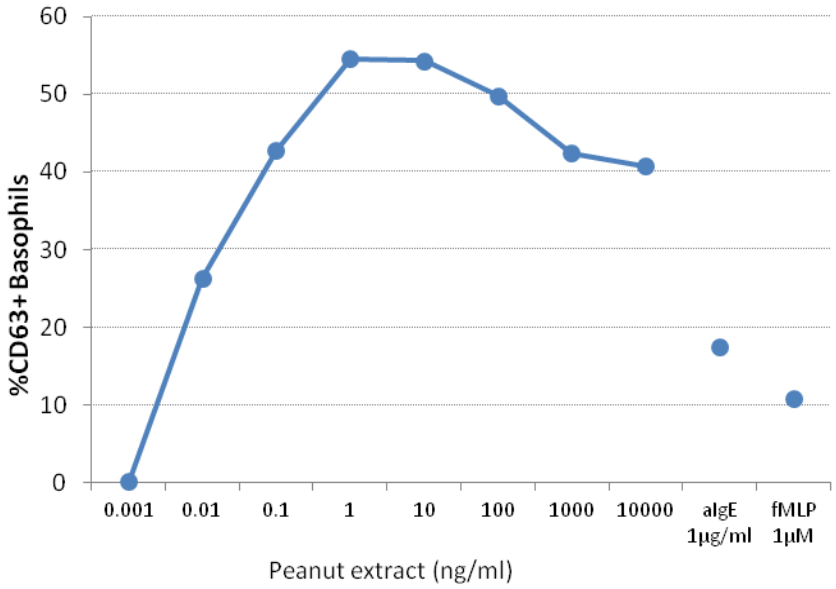


Figure 2.4 Dose-response curve of CD63 expression on the surface of basophils following stimulation with different concentrations of peanut extract.

Basophils were sensitised with plasma from a peanut allergic patient prior to stimulation with peanut extract. Anti-IgE and fMLP were used as positive controls. Abbreviations: aIgE, anti-IgE; fMLP, formyl-methionyl-leucyl-phenylalanine.

In the whole blood basophil activation assay, a range of concentrations of peanut extract from 0.01 to 10,000 ng/ml was initially tested but since no peanut allergic patient tested reacted at 0.01 ng/ml the range was reduced to 0.1 to 10,000 ng/ml in the final protocol.

2.3.6.6.3 Stimulation with purified peanut allergens

Passive sensitisation experiments were performed in which basophils sensitised with plasma from a peanut allergic patient were stimulated with purified peanut allergens, namely Ara h 1, Ara h 2, Ara h 3 and Ara h 6. Purified natural peanut allergens were individually able to activate basophils sensitised with plasma from PA patients with IgE to the respective allergen (Figure 2.5).

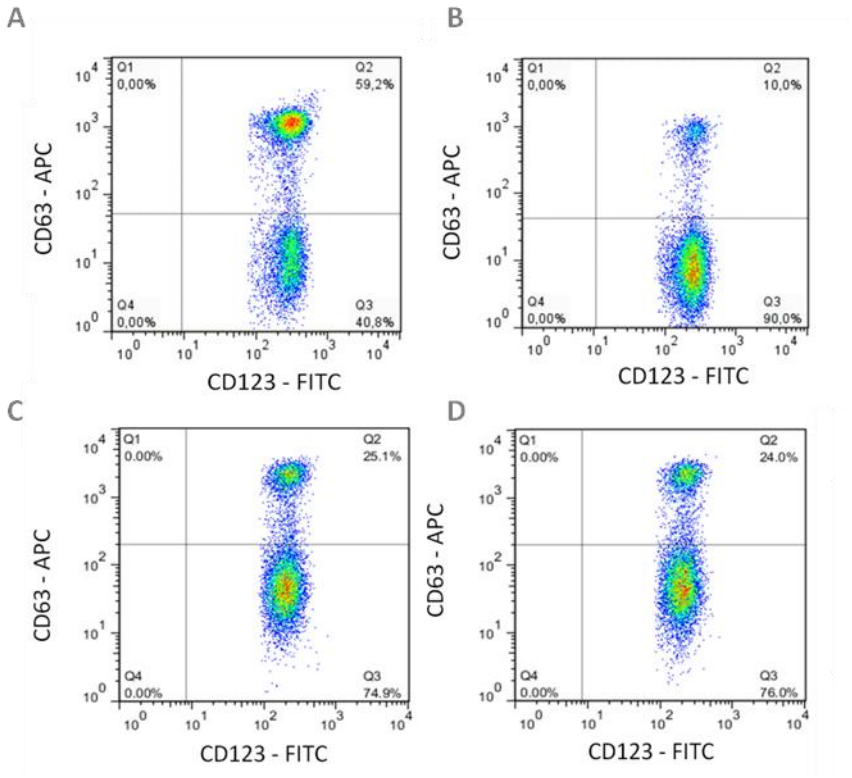


Figure 2.5 Purified native peanut allergens were able to individually induce basophil activation.

Upper right quadrants (Q2) show the percentage of CD63-positive basophils sensitised with plasma from a peanut allergic patient (with high levels of specific IgE to Ara h 1, Ara h 2 and Ara h 3) after stimulation with (A) 100 pM Ara h 1, (B) 50 pM Ara h 2, (C) 1 nM Ara h 3 and (D)1 nM Ara h 6.

2.3.6.6.4 IgE dependency

In a passive sensitisation basophil activation assay, basophils were separately sensitised with different dilutions (50%, 1%, 0.5% and 0.1%) of plasma from a peanut allergic patient in 0.5% HSA/RPMI. The concentration of peanut-specific IgE in undiluted plasma was 172 KU_A/l. Allergen-induced basophil activation occurred in a plasma dilution-dependent manner, possibly depending on the concentration of peanut-specific IgE – Figure 2.6.

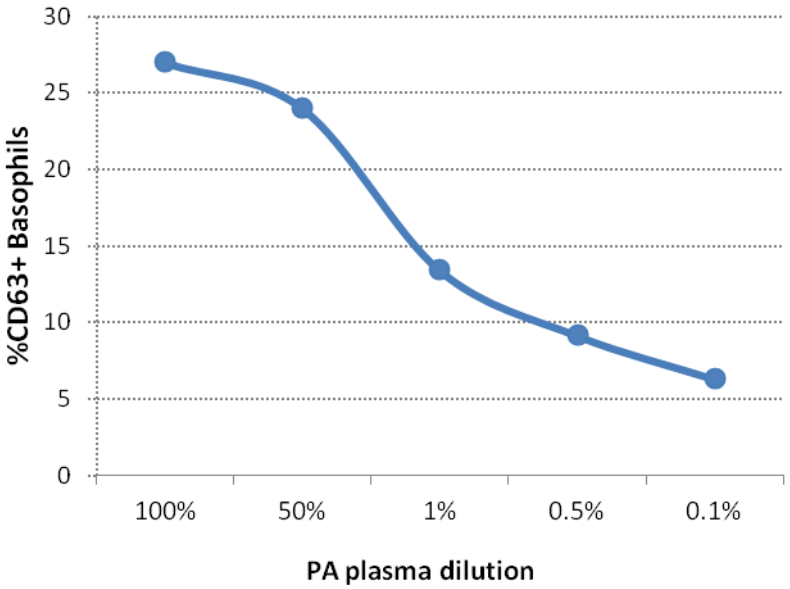


Figure 2.6 Peanut-induced CD63 expression on the surface of basophils sensitised with different concentrations of the same plasma sample of a peanut allergic patient (PA plasma).

Basophils were separately sensitised with undiluted plasma (100%) and serial dilutions (50%, 1%, 0.5% and 0.1%) of the same PA plasma and stimulated with 10 ng/ml peanut extract.

2.3.6.6.5 Donor dependency

2.3.6.6.5.1 Atopic versus non-atopic donors

Passive sensitisation experiments were performed using basophils from atopic and non-atopic donors sensitised with plasma from the same peanut allergic patient with high levels of peanut-specific IgE (172 KU_A/l) and stimulated with the same peanut extract and positive controls – Figure 2.7.

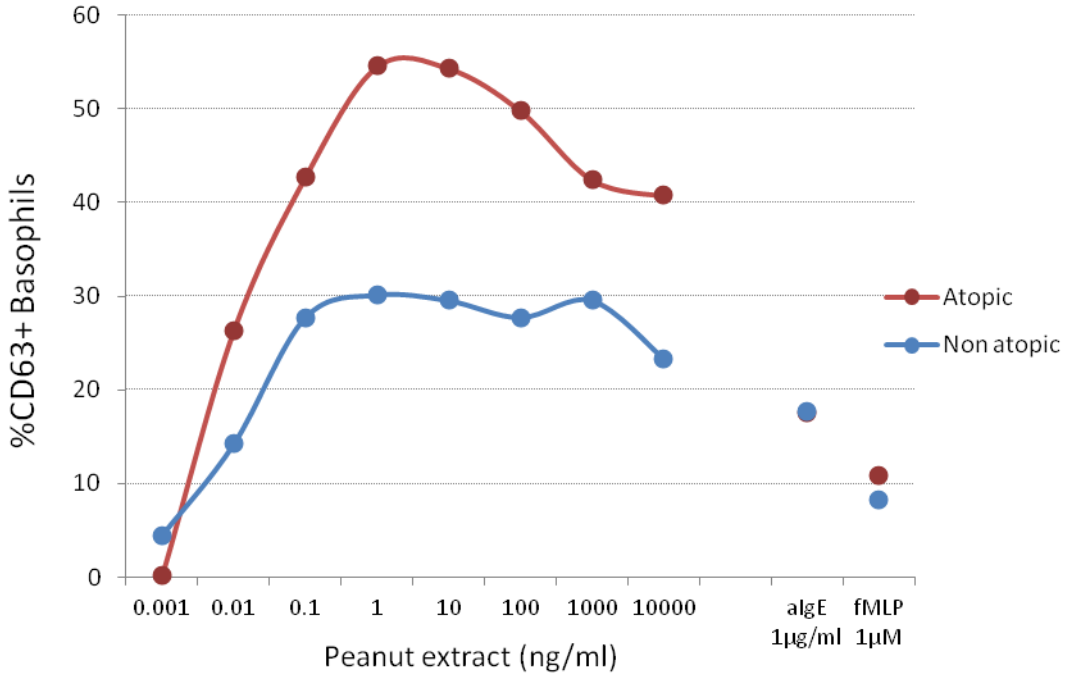


Figure 2.7 Peanut-induced CD63 expression of basophils from atopic and non-atopic donors sensitised with the same plasma sample of a peanut allergic patient.

Results for the atopic donor are presented in red and results for the non-atopic donor are presented in blue. Anti-IgE and fMLP were used as positive controls. Abbreviations: aIgE, anti-IgE; fMLP, formyl-methionyl-leucyl-phenylalanine.

Although sensitivity of basophils to peanut extract was similar, as evaluated by EC_{50} (approximately 0.01 ng/ml), there was lower basophil reactivity from the non-atopic donor, reaching a plateau at peanut extract concentrations ranging from 0.1 ng/ml to 10,000 ng/ml. This could be due to the lower density of FcεRI receptors on the surface of non-atopic donors' basophils[156]. Since the expression of FcεRI on the surface of basophils could be a limiting factor in passive sensitisation experiments, atopic (non-peanut allergic) volunteers were preferred as donors of cells for all subsequent passive sensitisation basophil experiments.

2.3.6.6.5.2 Variability in basophil responses among cell donors

In every passive sensitisation experiment, a set of cells was sensitised with plasma from the same peanut allergic patient (peanut-specific IgE of 172 KU_A/l, specific IgE to Ara h 1 of 74.1 KU_A/l, specific IgE to Ara h 2 of 77.6 KU_A/l and specific IgE to Ara h 3 of 33.6 KU_A/l), as an internal inter-assay control.

Figure 2.8 illustrates the heterogeneous response of basophils from different non-peanut allergic donors sensitised with the same plasma sample from a peanut allergic patient and stimulated with an optimal (10 ng/ml) and a suboptimal (0.1 ng/ml) concentration of peanut extract, as well as with anti-IgE (1 µg/ml).

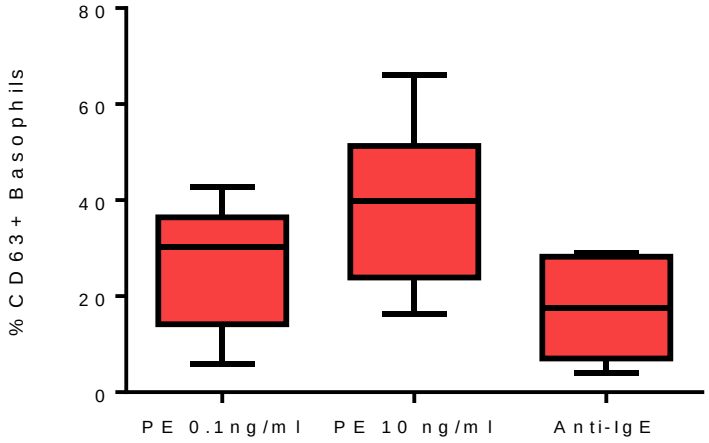


Figure 2.8 Variability of peanut-induced CD63 expression by basophils from different donors

Results are presented for basophils from 9 donors sensitised with the same plasma sample of a peanut allergic patient. Quartiles, maximum and minimum are represented. Abbreviations: PE, peanut extract.

2.3.7 Mast cell assays

To try to overcome the variability when using primary human basophils' from different blood donors and in view of obtaining a standardised *in vitro* passive sensitisation assay to investigate serological determinants of allergy and tolerance, mast cell assays were developed using the LAD2 mast cell line[157], kindly provided by Dr Dean Metcalfe at the Laboratory of Allergic Diseases in the NIH. The aim was to develop functional mast cell activation and inhibition flow cytometry-based assays to assess the functionality of peanut-specific IgE and other antibody isotypes in relation to peanut allergy versus tolerance.These assays had the additional advantage of allowing studying mast cells, which are the other main effector cells of IgE-mediated peanut allergy and anaphylaxis, apart from basophils.

LAD2 cells have been previously used in mediator release assays, where the concentration of mediators such as β-hexosaminidase is measured in the cell supernatant, and protocols have been published[158]. The result obtained is an average of the mediator released by single mast cells. The use of flow cytometry has the advantage of allowing analysing mast cell activation at

the single cell level. Lysosomal associated membrane proteins have been detected in human skin mast cells isolated from circumcision cutaneous tissue[159]. I hypothesised that LAD2 cells are able to bind IgE present in human plasma and to reproduce clinical reactivity (or tolerance) to peanut allergens by up-regulating (or not) the expression of these activation markers on their surface during degranulation measured by flow cytometry.

In pilot experiments, LAD2 cells expressed the high affinity IgE receptor and selected IgG receptors on their surface (Figure 2.9).

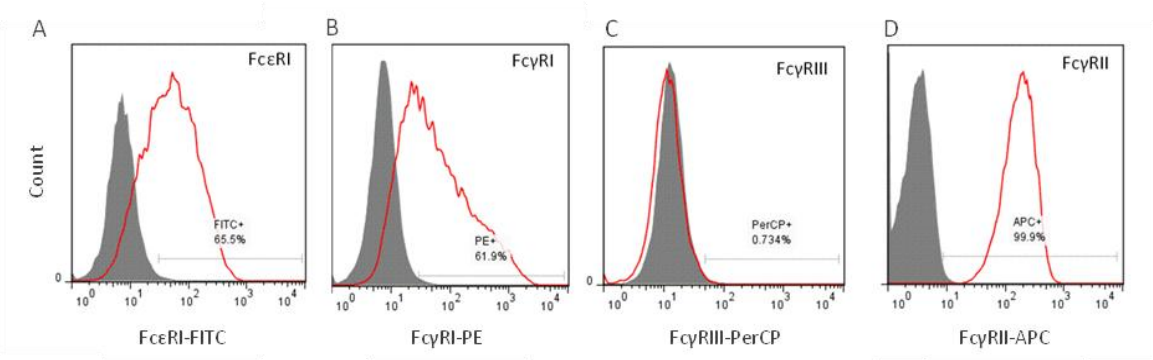


Figure 2.9 Expression of selected IgE and IgG receptors on the surface of LAD2 cells.

(A) high affinity IgE receptor, (B) CD64 or FcγRI, (C) CD16 or FcγRIII and (D) CD32 or FcγRII Histograms in red represent the mean fluorescent intensity compared to the isotype control in grey. The percentage of positive cells is represented.

LAD2 cells were sensitised with IgE from human plasma or myeloma (Figure 2.10) and up-regulated different degranulation markers after stimulation with 1 µg/ml of ionomycin (Figure 2.11).

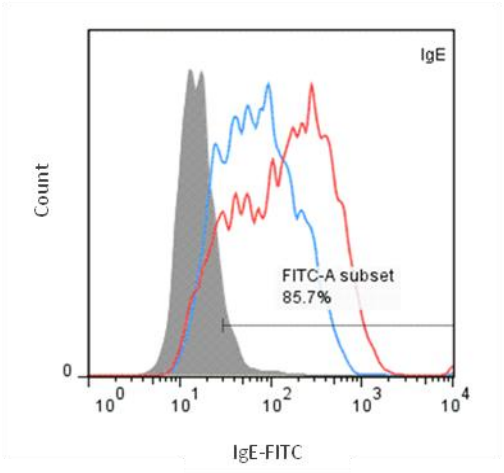


Figure 2.10 Expression of IgE on the surface of LAD2 cells

LAD2 cells were sensitised with plasma (red), human IgE purified from myeloma (blue) and buffer alone (grey). Histograms and percentage of positive cells are indicated.

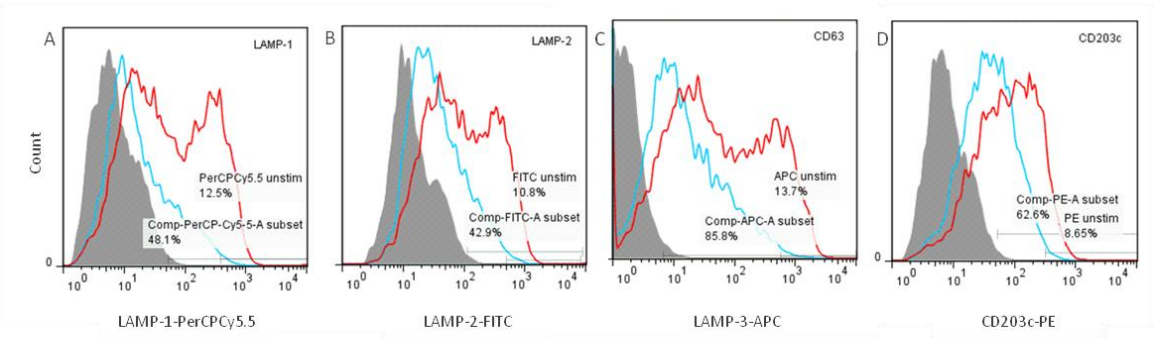


Figure 2.11 LAD2 cells up-regulate the expression of lysosomal-associated membrane proteins and CD203c following activation with ionomycin.

A. LAMP-1; B. LAMP-2; C. LAMP-3 or CD63; D.CD203c. Cells were stimulated with 1µg/ml ionomycin (red) or buffer alone (blue). Isotype control is shown in grey. Histograms and percentage of positive cells are indicated. Abbreviation: LAMP, lysosomal-associated membrane protein.

The mast cell activation assay consisted of a sensitisation phase, in which LAD2 cells were cultured in the presence of human plasma, and a stimulation phase, in which LAD2 cells sensitised with plasma were stimulated with peanut proteins and other IgE and non-IgE-mediated stimulants, as controls. Staining with IgE-PECy7 was used to control for sensitisation, i.e. for IgE binding. The changes in the expression of activation markers on the cell surface following stimulation were then evaluated using flow cytometry.

Up-regulation of LAMP-1 and LAMP-3 (CD63) - but not LAMP-2 - was observed on the surface of LAD2 cells that were previously sensitised with plasma from a peanut allergic patient following stimulation with peanut extract.

Sensitising LAD2 cells with same plasma sample from a PA patient resulted in consistent peanut-induced mast cell activation (Figure 2.12). This same plasma sample was used in all mast cell inhibition experiments as the reference plasma to assess the ability of test plasma to inhibit peanut-induced mast cell activation.

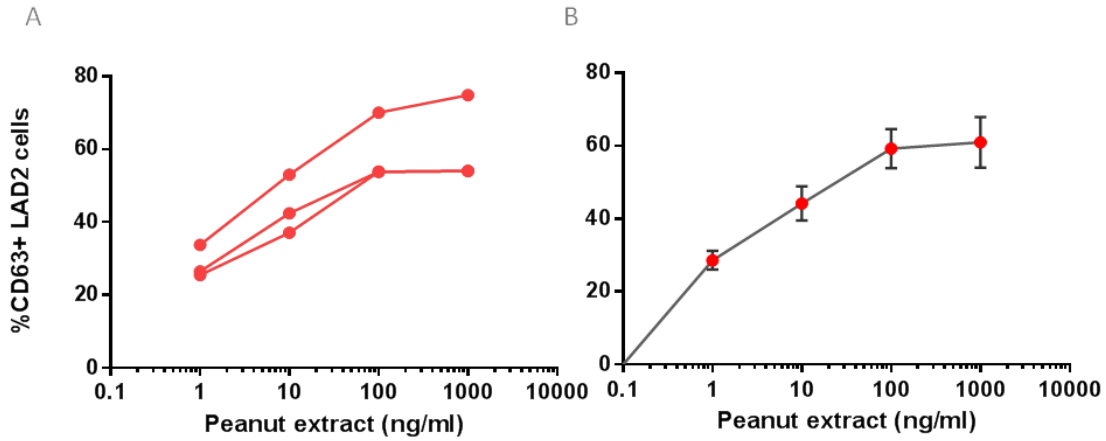


Figure 2.12 Dose-response of CD63 expression on the surface of mast cells sensitised with the same plasma sample of a peanut allergic patient following stimulation with different concentrations of peanut extract.

Results of 3 independent experiments are presented. A. Individual dose-response curves are represented. B. Mean and standard error are represented by dots and error bars, respectively.

2.3.7.1 Mast cell activation assay

LAD2 cells were cultured with rIL-4 for 5 days before overnight sensitisation with patients' plasma in a 1:10 dilution with culture media. Sensitised cells were stimulated with different concentrations ranging from 0.1 to 1000 ng/ml of peanut extract, 1 µg/ml of anti-IgE, 1 µg/ml of ionomycin in RPMI/BSA or 0.04% BSA RPMI for 30 minutes at 37˚C and stained with viability dye eFluor 450 (eBioscience, San Diego, CA, USA) and CD107b fluorescein isothiocyanate (clone ABL-93, eBioscience, San Diego, CA, USA), CD203c-phycoerythrin (clone NP4D6, Biolegend, San Diego, CA, USA), CD107a-peridinin chlorophyll protein-Cy5.5 (clone H4A3, Biolegend, San Diego, CA, USA), CD63-allophycocyanin (clone MEM259, Biolegend, San Diego, CA, USA) and IgE-phycoerythrin-Cy7 (clone NHE-18, Biolegend, San Diego, CA, USA) and analysed by flow cytometry. LAD2 cells were gated as CD203c+ viable cells. Mast cell activation was expressed as %CD107a+ or %CD63+ cells. Flow cytometry was performed on a FACS Canto II with FACSDiva software (BD Biosciences) and data was analysed using FlowJo software version 7.6.1 (TreeStar).

2.3.7.2 Inhibition of mast cell activation assay

For inhibition experiments, 20 µL of plasma from PS or NA patients or stimulation buffer was incubated with an equal volume of stimulation buffer and 10 µL of allergen (concentrations as indicated) at 37˚C for one hour. LAD2 cells sensitised overnight with 100µL of plasma from a PA patient (as described for the mast cell activation assay above), were washed and then added to the allergen-plasma mixture. The same PA plasma was used in all mast cell inhibition experiments. This plasma had similar IgE levels and patterns of sensitisation to peanut allergens (i.e. positive to Ara h 1, Ara h 2 and Ara h 3) to the plasma used in the basophil inhibition experiments: peanut-specific IgE of 255 KU_A/l, specific IgE to Ara h 1 of 84.4 KU_A/l, specific IgE to Ara h 2 of 125 KU_A/l, specific IgE to Ara h 3 of 1.8 KU_A/l.

2.3.7.3 Degranulation assay using RBP-SX38 cells

Cells of the RBL-SX38 rat basophilic cell line, which stably express human $Fc\varepsilon RI^{160}$, were used in the collaborative project described in Section 6.2. Cells were cultured in RPMI-1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich), 1% Penicillin-Streptomycin-Glutamine (Life Technologies, Carlsbad, Calif) and 1 mg/ml Genticin (Life Technologies). RBL-SX38 cells were harvested and resuspended in BAT buffer (HBSS with 2 mM $CaCl_2$) at $2x10^7$ cells/ml, then incubated with Phl p 7–specific IgE at a concentration of 0.5 mg/ml in 50 µl BAT buffer for 30 minutes at 37°C. Aliquots of 10^6 cells were washed with HBSS and incubated with 50 µl aliquots of the sera (diluted 1:2 in BAT buffer) for 30 minutes at 37°C. After further washing with HBSS, the cells were resuspended and incubated with 50 µl of 1 µg/ml of in-house produced, freshly biotinylated Phl p 7 diluted in BAT buffer for 30 minutes at 37°C. Surface-bound IgE was detected using anti-IgE-FITC (Vector Laboratories, Burlingame, Calif) and Phl p 7 binding to this IgE using streptavidin-allophycocyanin (BioLegend), with analysis by flow cytometry.

2.4 Statistical analyses

Statistical analyses were performed with SPSS 20.0 and STATA 12.1 for Windows, unless otherwise indicated. Significance was determined using a two-sided α level of 0.05.

2.4.1 Power calculation

The study to assess the performance of the basophil activation test to diagnose peanut allergy was undertaken in a specialised tertiary allergy referral centre where the anticipated prevalence of peanut allergy is around 50%. Based on a previous study[143], the expected sensitivity and specificity of BAT is 95%. Our desired precision in estimating 95% confidence intervals was 6.5%. To achieve this precision for sensitivity and specificity, we need a total sample size of 89 patients[161]. Taking into account that BAT non-responders cannot be analysed and that the rate of BAT non-responders is usually between 7.5-10%, this resulted in a final sample size estimation of 100 patients.

2.4.2 Comparison of groups

Qualitative variables were expressed as number and percentage and compared between PA and PS using the Fisher's Exact test or the Chi-Square test. Quantitative continuous variables were expressed as median and interquartile range and compared using the Mann-Whitney U test or the Kruskall-Wallis test. For comparison of paired samples, the Wilcoxon Signed Ranks Test was used.

2.4.3 Diagnostic study

The diagnostic performance of the whole blood basophil activation test to peanut was assessed and diagnostic cut-offs were determined. The utility of the basophil activation test in combination with other diagnostic tests was also assessed.

2.4.3.1 ROC curve analyses

The performance of allergy tests in predicting the allergic status was examined using ROC curve analyses. The cut-offs to predict peanut allergy and peanut tolerance for BAT and the various allergy tests with optimal accuracy were determined according to the Youden index and validated. Internal validation was performed using repeated random sub-sampling validation (bootstrap) and "leave-one-out" methodologies[162]. Both methodologies produced similar results in estimating the optimal cut-points and the former methodology is reported. The 95% confidence interval was constructed using bootstrapping methodology with 1000 replications to reflect on the reproducibility[163]. An external validation study was also conducted using a new cohort of 65 subjects (25 PA, 24 PS and 16 NA) mainly recruited from the PAS (Peanut Allergy Sensitisation) study, a group of patients from all over the country that were excluded from the LEAP study[8], and from a private Paediatric Allergy clinic in London. The cut-offs previously determined in the primary study population were applied to this validation study population and sensitivity, specificity, predictive values, likelihood ratios and accuracy were calculated.

2.4.3.2 Agreement analyses

Three Paediatric Allergy specialists were asked to classify 44 equivocal cases from the primary study population as peanut allergic or tolerant based on the history and results of SPT, peanut-specific IgE and specific IgE to Ara h 1, Ara h 2, Ara h 3, Ara h 8 and Ara h 9. The agreement between physicians was calculated as percentages and assessed with κ statistics[164].

2.4.3.3 Combination of the basophil activation test with other diagnostic tests

In the primary study population, following ROC curve analyses, the performance of BAT with SPT, peanut-specific IgE and Ara h 2-specific IgE was compared using conventional cut-offs, indicated in Figure 1.6. For skin prick test, 8 mm and 3 mm were used as positive and negative cut-offs, respectively[129]. For specific IgE to peanut, 15 KU_A/L and 0.35 KU_A/L were used as positive and negative cut-offs, respectively[128, 129]. For specific IgE to Ara h 2, 1 KU_A/L and 0.10 KU_A/L were used as positive and negative cut-offs, respectively[44]. For BAT, the cut-off for the mean of percentage of CD63-positive basophils at 10 and 100 ng/ml of peanut extract was used as both positive and negative cut-off. The diagnostic utility of BAT was further assessed when considered

in combination with other allergy tests, i.e. considering the results of different tests simultaneously, and when considered as a second or third step in the diagnostic process, i.e. performed in selected patients where the results of single or of combinations of tests were equivocal.

When interpreted individually, the results of standard allergy tests were considered diagnostic of allergy when ≥95% PPV cut-off, diagnostic of tolerance when <95% negative predictive value (NPV) cut-off and equivocal when between the positive and the negative cut-offs (Figure 1.6). BAT was considered equivocal in the case of "non-responders" (defined as <5% CD63-positive basophils to IgE-mediated controls and ≥5% CD63%+ basophils to fMLP).

The combination of allergy tests were interpreted as equivocal if one test result was ≥95% PPV cut-off and another test result was <95% NPV cut-off or when all tests gave equivocal results (as defined above) or a combination of equivocal results and results <95%NPV.

In these simulations, oral food challenges were deemed necessary when the interpretation of tests was equivocal. The combination of SPT and peanut-specific IgE was the clinical reference-point against which the change in the number of oral food challenge required was determined.

2.4.4 Severity and threshold study

Since BAT was shown to discriminate between peanut allergic and peanut tolerant patients with high diagnostic accuracy[165], I hypothesised that BAT may also indicate the severity and the threshold of allergic reactions to peanut in allergic patients.

2.4.4.1 Logistic regression analyses

Allergy test parameters noted to have differences (p value <0.2) in the comparison of groups (i.e. severe versus non-severe reactors and low versus high threshold) were further tested as independent variables in logistic regression analyses using severity or threshold dichotomised groups as dependent variables. Since several (correlated) BAT parameters distinguished between severity and threshold groups, only the best performing BAT parameter (based on the lowest p value) was tested in the logistic regression analysis. For severity this parameter was the ratio of the percentage of CD63-positive basophils at 100 ng/ml of peanut to the percentage of

CD63-positive basophils following stimulation with anti-IgE (CD63 peanut/anti-IgE). For threshold this parameter was CD_{sens} determined using CD63. Independent associations between severity or threshold and allergy test parameters were further investigated by forward multivariable logistic regression analyses and only variables significantly contributing to the model ($p<0.05$) were retained.

2.4.4.2 BAT cut-offs for severity and for threshold of allergic reactions

In order to quantify the differences in basophil activation between severity and threshold groups, patients were dichotomised based on the 75th percentile of CD63 peanut/anti-IgE or CD_{sens} and the proportion of patients with severe reactions or low threshold were compared between those falling above and below the 75th percentile.

BAT cut-offs for severity and threshold were also determined by ROC curve analyses, using the software MedCalc 13.3 (MedCalc Software, Ostend, Belgium). The Youden index was the criteria adopted to select the optimal cut-offs. Bias-corrected and accelerated bootstrapped 95% confidence intervals were calculated for sensitivity, specificity and predictive values.

2.4.4.3 Correlation between severity and threshold

The correlation between the clinical and the BAT parameters for severity and threshold were assessed using Spearman correlation.

2.4.5 IgG4 study

Apart from the analyses for comparison of groups, described in section 2.4.2. above, in the work described in chapter 7 referred to here as "IgG4 study", additional statistical methods were used, including analysis of covariance using ranks and multivariate logistic regression analysis.

2.4.5.1 Analysis of covariance using ranks

In order to confirm whether the peanut allergic status in PA and PS patients could be explained by differences in the peanut-specific IgG4/IgE ratio or differences in peanut-specific IgE, the peanut-

specific IgG4/IgE ratios was adjusted for peanut-specific IgE by analysis of covariance using rank transformation[166].

2.4.5.2 Multivariate logistic regression model

To clarify the contribution of peanut-specific IgE and of peanut-specific IgG4 to the clinical outcome in PA and PS patients, a multivariate logistic regression model with log base 10 transformed peanut-specific IgG4 and peanut-specific IgE as well as a relative importance analysis were performed using JMP® Pro 11.2.1 for Windows.

2.4.5.3 Correlation analyses

To study the relationship between the ratio of peanut-specific IgG4 to IgE and basophil activation, the Spearman correlation coefficient was calculated and the corresponding p value determined.

Chapter 3 Gating strategy for the whole blood basophil activation test

Different cell-surface markers can be used for the identification of basophils in whole blood, including the combination of CD123 and HLA-DR[64, 69]. CD123 is the low affinity (α) subunit of the IL-3 receptor and is highly expressed on plasmacytoid dendritic cells and basophils, and in low levels on monocytes, eosinophils, myeloid dendritic cells and haematologic progenitor cells. While eosinophils can be excluded by side-scatter, additional staining with HLA-DR is required to discriminate between HLA-DR-negative basophils and HLA-DR-positive dendritic cells and monocytes. In previous studies, the expression of CD123 and HLA-DR were shown to be stable with the atopic status of patients and following basophil activation[66, 69]. However, stimulation of the IL-3 receptor by IL-3 can increase the baseline expression of CD203c and possibly CD63 and maximise the up-regulation of CD63 upon basophil activation[70, 167]. Therefore, I hypothesised that CD123 expression could change in response to basophil activation. With increasing attention given to basophils in the coordination of adaptive immune responses and their possible role in antigen presentation[168-170], I hypothesised that there could be an increase in the expression of HLA-DR by basophils following activation by allergen or by other stimulants. Given the important implications for the gating strategy to be adopted for BAT in future studies, I sought to determine whether the expression of CD123 and HLA-DR remained unchanged with basophil activation and to select the best gating strategy using these markers.

3.1 Summary of methodology

Basophil activation test to peanut was performed in children aged from 5 months to 17 years, as described in Section 2.3.6.1. Basophil activation was expressed as the proportion of CD63-positive basophils, corrected for the negative control, and as a ratio of the MFI of CD203c-PE of stimulated to unstimulated basophils, the stimulation index of CD203c (SI CD203c). The variation of CD123 was defined as the proportion of the difference between the MFI of CD123-FITC of the negative control and of the stimulated cells and the MFI of CD123-FITC of the negative control and calculated using the formula:

Variation of CD123-FITC = (MFI CD123-FITC pre-stimulation - MFI CD123-FITC post-stimulation)/MFI CD123-FITC pre-stimulation.

All patients tested (n=116) responded to fMLP; therefore, when assessing basophil activation induced by fMLP all patients were considered (n=116). When assessing basophil activation induced by anti-IgE (n=104), patients with non-responder basophils, i.e. basophils which did not respond to any IgE-mediated stimulants but only to fMLP, were excluded. When evaluating the response to peanut, peanut allergic patients (as defined in Section 2.1) with responding-basophils were considered (n=42). For data analysis, when only one concentration of peanut extract was used, the optimal concentration of 100 ng/ml was selected.

Qualitative variables were represented as numbers of patients and percentage (taking into account the missing values) and groups were compared using the Fisher's Exact test or Chi-Square test, as appropriate. Continuous variables were represented as median and range and were compared using the Mann-Whitney U test or the Kruskall-Wallis test, as appropriate. Wilcoxon-signed rank test was used to compare samples before and after stimulation. For receiver-operating characteristic (ROC) curve analysis, the performance of the average percentage of CD63-positive basophils at 10 and 100 ng/ml of peanut extract determined using different gating strategies was evaluated against the patients' allergic status to peanut, i.e. in relation to allergy versus tolerance. All statistical analyses were performed with SPSS 20.0 for Windows. Significance was determined using a two-sided α level of 0.05.

3.2 Gating on basophils using CD123 and HLA-DR led to the loss-to-analysis of cells

Identifying basophils with CD123 and HLA-DR led to the loss to analysis of cells particularly in conditions where basophils were activated. The baseline number of unstimulated basophils was variable (median=1721, IQR=1225-2184) but comparable between atopic and non-atopic patients (p=0.444) and between peanut allergic and peanut tolerant children (p=0.739). Following basophil stimulation with fMLP (p=0.012) and anti-IgE (p=0.005), the number of basophils was significantly reduced compared to baseline (Table 3.1). Of note, if only patients with non-responder basophils were considered (n=12), the change in basophil number was significant after fMLP stimulation (p=0.004) but not after anti-IgE stimulation (p=0.099), suggesting that the reduction in cell number was dependent on basophil activation.

Table 3.1. Number of basophils (gated as SSC^{low} CD123+ HLA-DR- cells) in different stimulation conditions.

Median and interquartile range are represented. p value refers to the comparison of post-stimulation conditions with the negative control. Significant p values are marked in bold.

Stimulant	n	Pre-stimulation	Post-stimulation	p value
fMLP	116	1722 (1226, 2184)	1572 (1037, 2100)	**0.012**
Anti-IgE	104	1722 (1221, 2191)	1414 (972, 1995)	**0.005**
Peanut extract	42	1732 (1212, 2174)	1514 (914, 2041)	0.134

Considering anti-IgE stimulation, 14% of patients showed more than 25% decrease in the number of basophils compared to the negative control (Figure 3.1). In 27% of patients, this number decreased to below 1000 basophils. Selecting peanut allergic patients, a trend was seen toward a reduction in the basophil number after stimulation with peanut extract compared to the negative control (n=42, p=0.081). As the starting volume of blood, and thus the starting number of cells, was similar in all experimental conditions, I hypothesised that the expression of the identification markers, CD123 and/or HLA-DR, changed with basophil activation.

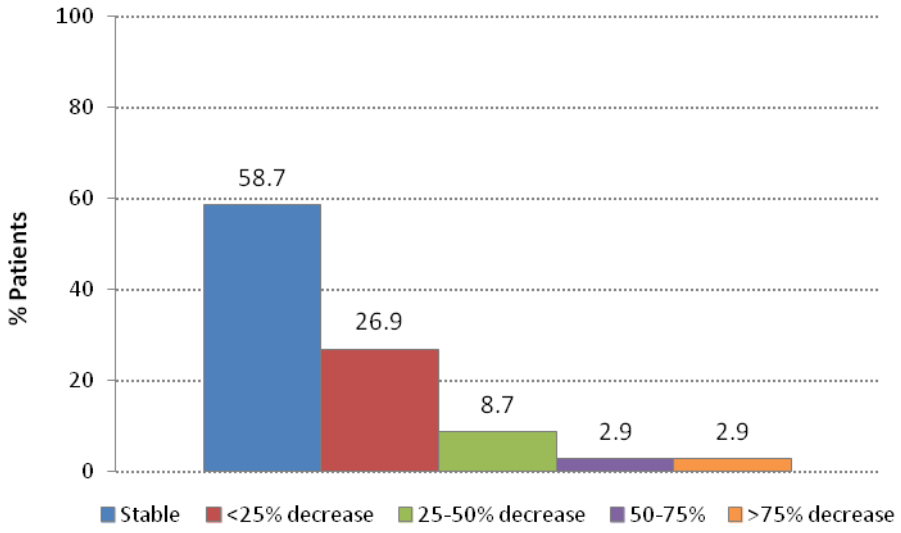


Figure 3.1 Variation in the number of basophils following stimulation with anti-IgE compared to unstimulated basophils.

N=104. Variation was calculated as (MFI CD123-FITC pre-stimulation - MFI CD123-FITC post-stimulation)/MFI CD123-FITC pre-stimulation.

3.3 CD123 is down-regulated with basophil activation

To evaluate the changes in the expression of CD123 and HLA-DR on the surface of basophils, I used CD203c to gate on the basophil population (Figure 3.2).

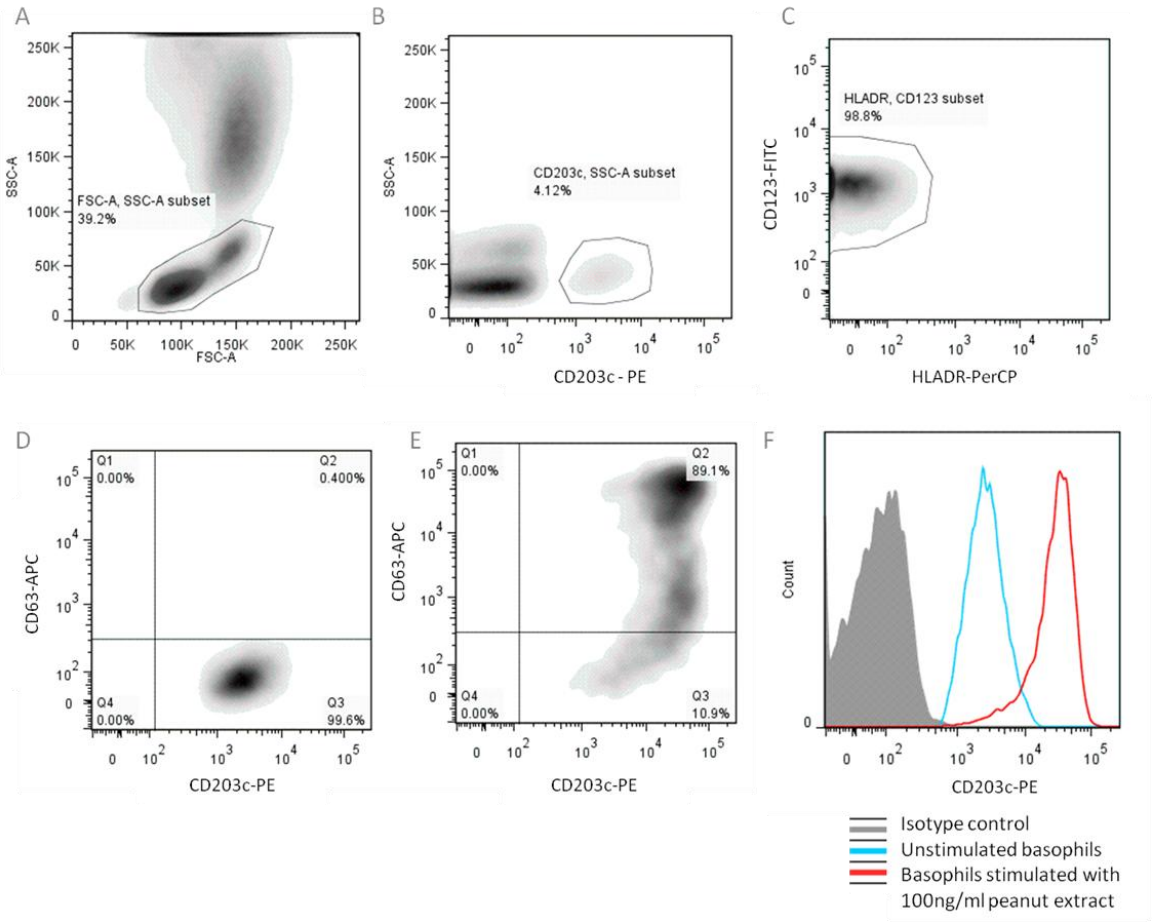


Figure 3.2 Preferred gating strategy for the basophil activation test.

Basophils were identified in whole blood in the lymphocyte-monocyte area (A) as SSC^{low}/CD203c+ (B) and CD123+/HLA-DR- (C). Figures D and E show CD63 and CD203c expression in unstimulated basophils (D) and basophils stimulated with 100 ng/ml of peanut extract (E). In figure F, histograms show the changes in the mean fluorescence intensity of CD203c-PE following stimulation with peanut extract.

The baseline MFI of CD123-FITC was variable between patients (median=1082, interquartile range=98-1549) but comparable between atopic and non-atopic children (p=0.153) and between peanut allergic and peanut tolerant patients (p=0.826). Down-regulation of CD123 by basophils was seen following stimulation with fMLP (n=116, p<0.001), anti-IgE (n=104, p<0.001) and peanut extract (n=42 peanut allergic patients, p<0.001) - Table 3.2, Figure 3.3.A. and Figure 3.4.A. In 92.3% of patients, anti-IgE stimulation led to a decrease in the MFI of CD123-FITC compared to

the negative control: in 38.5% less than 25% decrease, in 26.9% between 25 and 50% decrease, in 13.5 % between 50 and 75% decrease and in 13.5% greater than 75% decrease (Figure 3.4.B). The down-regulation of CD123 expression on the surface of basophils stimulated by fMLP and by anti-IgE was correlated (r_s=0.723, p<0.001), suggesting this phenomenon happened in the same patients with different stimulants. The decrease in CD123 expression with anti-IgE stimulation was similar between atopic and non-atopic (p=0.828) and between peanut allergic and peanut tolerant children (p=0.431). The expression of CD123 was stable when basophils were not activated - for example, after stimulation with peanut in peanut tolerant patients (p=0.658) or after stimulation with anti-IgE in non-responders' basophils (p=0.083), while down-regulation was still observed in this subgroup after stimulation with fMLP (p=0.006).

Table 3.2 Expression of CD123 on the surface of basophils (gated as SSC^{low} CD203c+ cells) as measured by MFI of CD123-FITC in different stimulation conditions.

Median and interquartile range are represented. p value refers to the comparison of post-stimulation conditions with the negative control.

Stimulant	n	Pre-stimulation	Post-stimulation	p value
fMLP	116	1082 (98, 1549)	514 (75, 1020)	<0.001
Anti-IgE	104	1081 (95, 1549)	495 (60, 1111)	<0.001
Peanut extract	42	1172 (80-2058)	293 (40, 1244)	<0.001

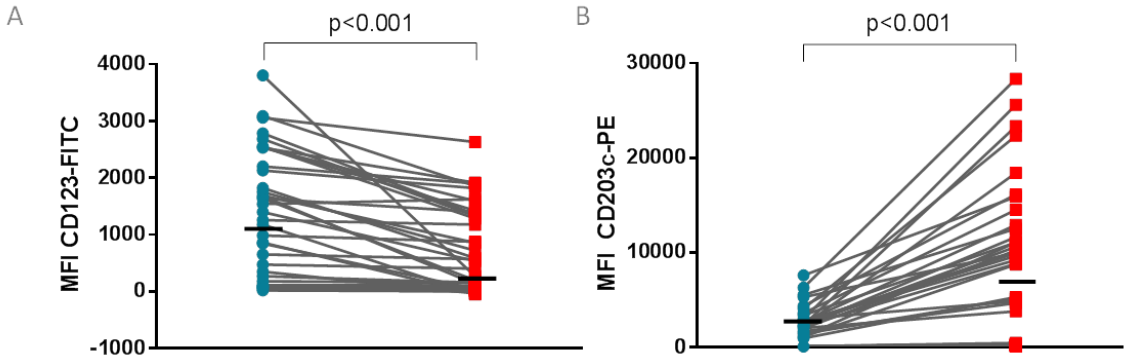


Figure 3.3 Changes in the MFI of CD123-FITC and of CD203c-PE following stimulation with peanut extract.

A. MFI of CD123-FITC; B. MFI of CD203c-PE; in unstimulated basophils (blue) and following stimulation with 100 ng/ml of peanut extract (red). n=42 peanut allergic patients.

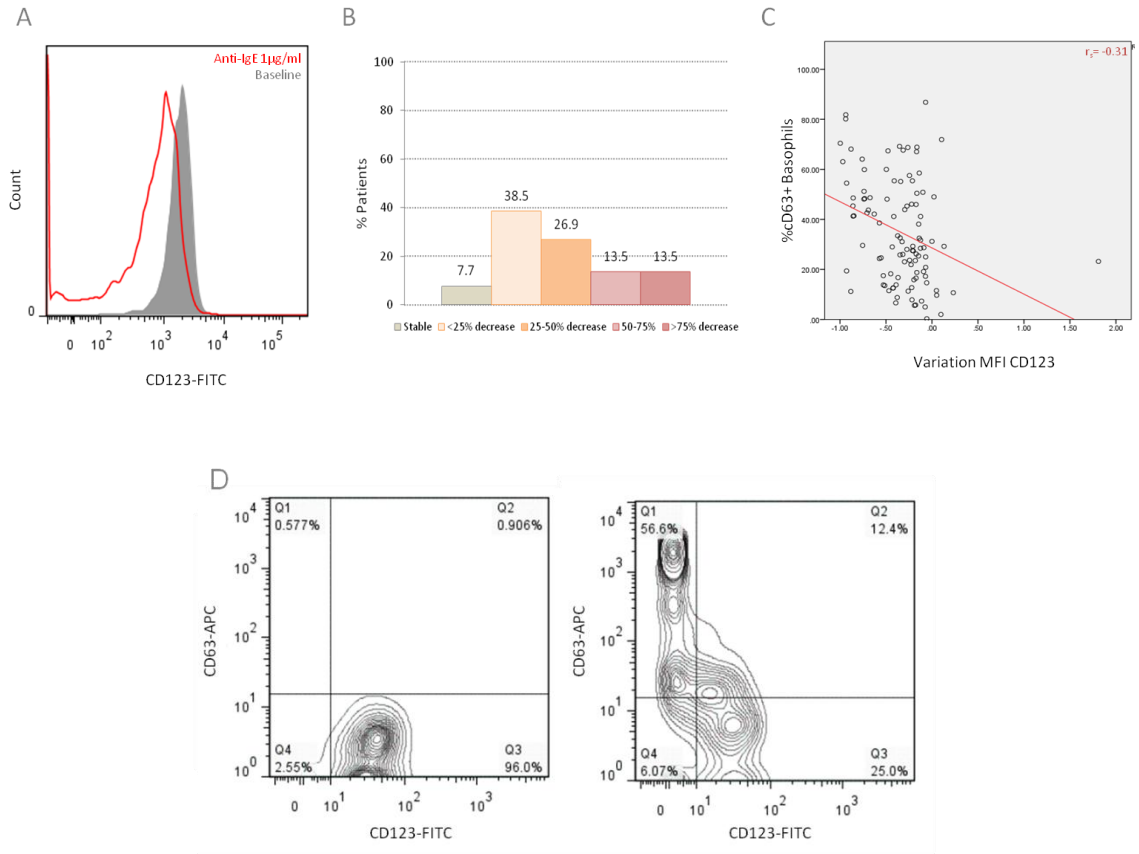


Figure 3.4 Down-regulation of CD123 expression with basophil activation.

A. Histogram of MFI of CD123-FITC before and after anti-IgE stimulation; B. Change in the MFI of CD123-FITC on the surface of basophils following anti-IgE stimulation; C. Correlation between the decrease in CD123 and the up-regulation of CD63 on the surface of basophils; D. Relationship between CD63 and CD123 on CD203c+ cells at rest and with activation from a donor demonstrating the loss of CD123 expression phenotype in activated cells.

Basophils were HLA-DR-negative and were distinct from the CD123+ HLA-DR+ population probably constituted by plasmacytoid dendritic cells. Following basophil activation, the HLA-DR expression did not increase and remained distinct from HLA-DR-positive cells.

3.4 Decrease in CD123 correlates with the up-regulation of CD63 and CD203c

Taken together the previous observations indicate that the down-regulation of CD123 by basophils is an activation-dependent phenomenon. A significant correlation was observed between the decrease in the MFI of CD123 and the up-regulation of basophil activation markers after stimulation with anti-IgE (Figure 3.4.C) as measured by the stimulation index of CD203c (r_s=-

0.35, p<0.001) or by the percentage of CD63-positive basophils (r_s=-0.31, p<0.001), suggesting that the basophils that down-regulate CD123 the most are also the ones that express more CD63 and CD203c. The correlations between changes in the MFI of CD123-FITC and in the percentage of CD63-positive basophils or the SI of CD203c following stimulation with fMLP (-0.191, p=0.04 and -0.223, p=0.016, respectively) and following stimulation with peanut extract (-0.229, p=0.145 and -0.31, p=0.051, respectively) were less strong.

3.5 Additional use of CD203c prevented the loss-to-analysis of activated basophils

The down-regulation of CD123 with basophil activation has important implications in gating strategies that depend on the marker CD123. Identifying basophils using CD123 and HLA-DR led to the loss-to-analysis of basophils, particularly of the ones with higher expression of the activation markers CD63 and CD203c, and thus leading to an underestimation of basophil activation (Table 3.3 and Figure 3.5.A). Adding CD203c as an identification marker maintained the cell number, regardless of the basophil activation status. Thus, the basophils that were most highly activated were included in the analysis, improving the value of the test (Table 3.3 and Figure 3.5.B). The expression of CD203c remained stable or increased following basophil activation, allowing a distinct separation from the remaining blood cells (Figure 3.3.B.).

Table 3.3 Comparison of different gating strategies to identify basophils.

Median and inter-quartile range are represented. Medians were compared between 3 groups using Kruskall-Wallis test and between 2 groups using Mann-Whitney U test: [1]CD123+ HLA-DR- versus CD203c+; [2]CD203c+ versus CD203c+ HLA-DR-, [3]CD123+ HLA-DR- versus CD203c+ HLA-DR-. [a] n=116 (whole population), [b] n=104 (non-responders were excluded), [c] n= 42 (peanut allergic). Abbreviations: MFI, mean fluorescence intensity; SI, stimulation index; fMLP, formyl-methionyl-leucyl-phenylalanine.

Parameters	Stimulants	CD123+ HLA-DR-	CD203c+	CD203c+ HLA-DR-	p value	p value[1]	p value[2]	P value[3]
Number of basophils	Negative control[a]	1722 (1226, 2184)	1782 (1334-2239)	1697 (1268-2134)	0.635	0.390	0.436	0.908
	Anti-IgE[b]	1414 (972-1995)	2156 (1620-3097)	1939 (1452-2877)	**<0.001**	**<0.001**	0.06	**<0.001**
	fMLP[a]	1572 (1037, 2100)	2146 (1684-2940)	1891 (1447-2715)	**<0.001**	**<0.001**	0.084	**<0.001**
	Peanut extract[c]	1514 (914-2041)	2351 (1748-3062)	2104 (1615-2752)	**<0.001**	**<0.001**	0.348	**0.001**
%CD63+ basophils	Anti-IgE[b]	24.8 (10.7, 42.5)	29.4 (17.3-48.9)	32.0 (17.1-53.9)	**0.021**	**0.020**	0.809	**0.013**
	fMLP[a]	28.2 (15.9, 42.3)	41.1 (26.3-53.4)	41.4 (26.9-56.9)	**<0.001**	**<0.001**	0.644	**<0.001**
	Peanut extract[c]	32.3 (10.9, 56.2)	41.0 (18.0-56.9)	42.1 (20.0-68.5)	0.185	0.220	0.561	0.074
MFI CD63-APC	Anti-IgE[b]	104.0 (26.3-195.2)	148 (36, 345)	159.3 (45.4-350.8)	**0.019**	**0.029**	0.611	**0.009**
	fMLP[a]	139 (37, 284)	285 (76, 683)	307 (81, 708)	**<0.001**	**<0.001**	0.678	**<0.001**
	Peanut extract[c]	138.4 (24.8-270.7)	190 (37, 513)	202.9 (42.6-567.4)	0.267	0.271	0.629	0.113
SI CD203c	Anti-IgE[b]	2.4 (1.5-3.8)	2.9 (2.0-4.2)	3.2 (2.0-4.6)	**0.004**	**0.028**	0.191	**0.002**
	fMLP[a]	2.3 (1.7-3.1)	2.6 (2.2-3.7)	2.8 (2.2-4.1)	**<0.001**	**0.001**	0.280	**<0.001**
	Peanut extract[c]	3.3 (1.8-5.3)	3.8 (2.4-4.8)	4.3 (2.4-5.4)	0.193	0.386	0.260	0.088
MFI CD203c-PE	Anti-IgE[b]	2331 (197-5633)	4833 (254, 9020)	5013 (29-9161)	**0.008**	**0.011**	0.677	**0.005**
	fMLP[a]	2763 (216-5139)	4549 (279, 7961)	4908 (334-8270)	**0.001**	**0.002**	0.813	**0.001**
	Peanut extract[c]	3631.6 (179.4-8491.4)	5312 (206, 11976)	7012 (236-11857)	0.173	0.140	0.714	0.083

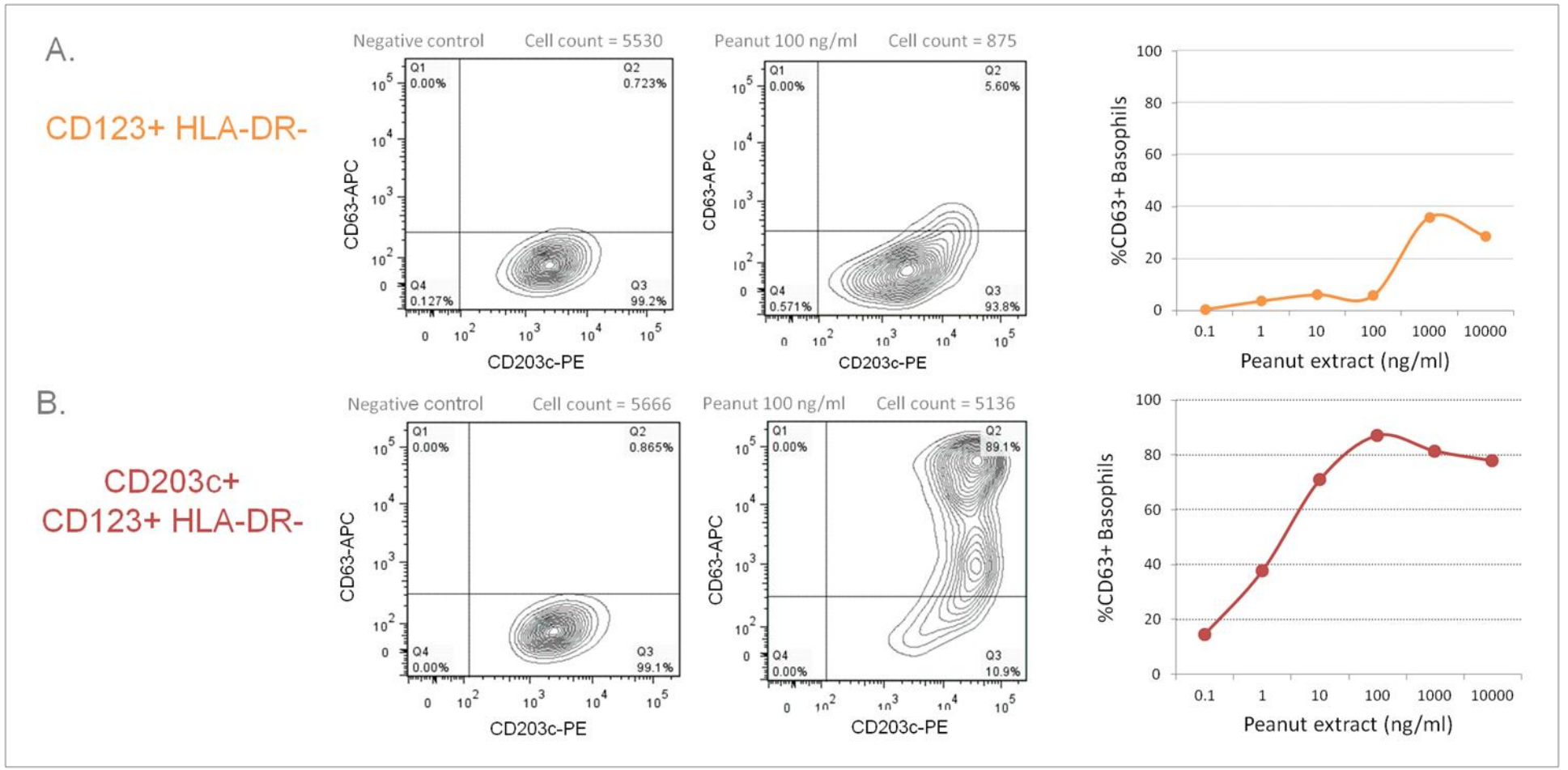


Figure 3.5 Gating with CD123/HLA-DR led to the loss-to-analysis of basophils and underestimation of basophil activation.

In a representative experiment, basophils were gated as SSClow CD123+ HLA-DR- (A) or SSClow CD203c+ HLA-DR- (B). The percentage of CD63+ basophils is represented in different conditions: negative control, peanut extract 100ng/ml and peanut-dose-response curve. Note the bi-exponential display[171].

3.6 Selecting the optimal gating strategy using CD203c

In a subset of patients (16%), gating solely on SSC^{low} CD203c+ cells included a population of cells that were HLA-DR+, which in selected experiments were identified as CD14+. To overcome this, three identification markers were combined to gate on basophils in subsequent experiments: SSC^{low} CD203c+ cells were first selected and then gated on CD123/HLA-DR following the contour of the cell population of interest, including $CD123^{low}$ as well as $CD123^{high}$ cells, all HLA-DR-.

The gating strategy using CD203c+ HLA-DR- proved superior to CD123+ HLA-DR-, similar to what was observed for gating on CD203c+ cells alone (Table 3.3). The combination of the three markers was superior to CD203c+ in the subgroup of peanut allergic patients improving the detection of basophil activation (Table 3.3, borderline non-significant p values for the percentage of CD63-positive basophils, SI CD203c and MFI of CD203c) and in patients with a subset of CD203c+ HLA-DR+ cells mentioned above thus avoiding contamination with HLA-DR+ cells.

Markers for other immune cells (anti-CD14, anti-CD3, anti-CD19, anti-CD41 and in six of these experiments also anti-CD56) were included in selected experiments (n=10) to exclude potential cell contamination within the adopted basophil gate (Figure 3.6). The results were similar using the three gating strategies: SSC^{low}/CD203c+(Figure 3.6.C), SSC^{low}/CD203c+/HLA-DR- (Figure 3.6.B) and SSC^{low}/lineage-negative/CD203c+/HLA-DR- (Figure 3.6.A); however, using all the markers (Figure 3.6.A) a "cleaner" population was obtained. None of the other cell populations (CD14+, CD3+, CD19+, CD41+, CD56+) expressed simultaneously both markers CD203c or CD63 (Figure 3.6.D and E). Given the higher cost and longer time involved in the use of multiple markers, the use of CD203c, CD123 and HLA-DR was preferred in subsequent experiments.

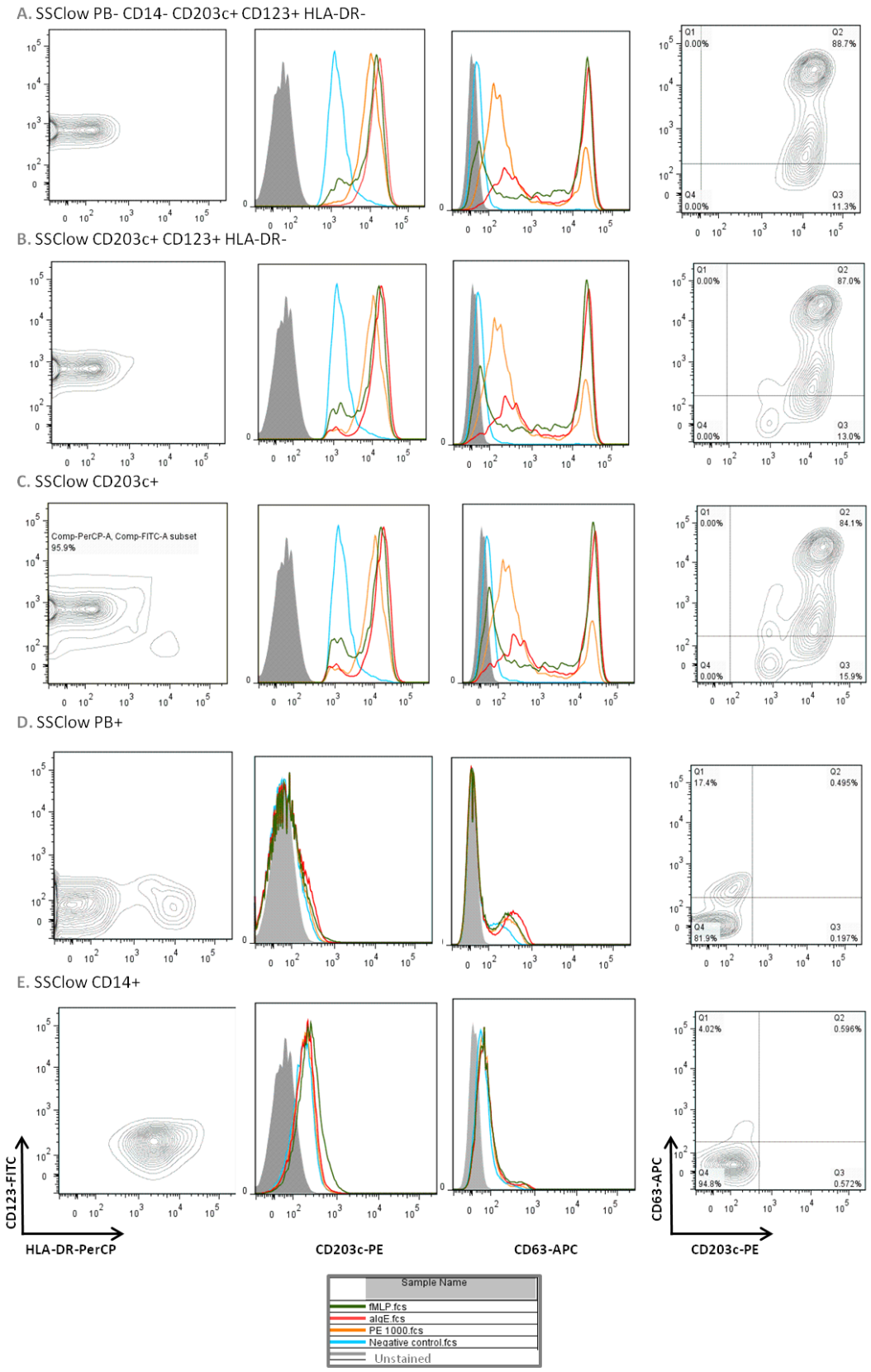


Figure 3.6 Representative experiment of basophil activation performed with additional staining for CD3+, CD19+, CD41+, CD56+ and CD14+ using antibodies labelled with pacific blue (PB) to exclude possible cell contamination within the adopted basophil gating strategy.

Histograms represent unstained cells (grey) and cell stimulated with buffer alone (blue), 1000 ng/ml of peanut extract (orange), 1 µg/ml of anti-IgE (red) or 1 µM of fMLP (green).

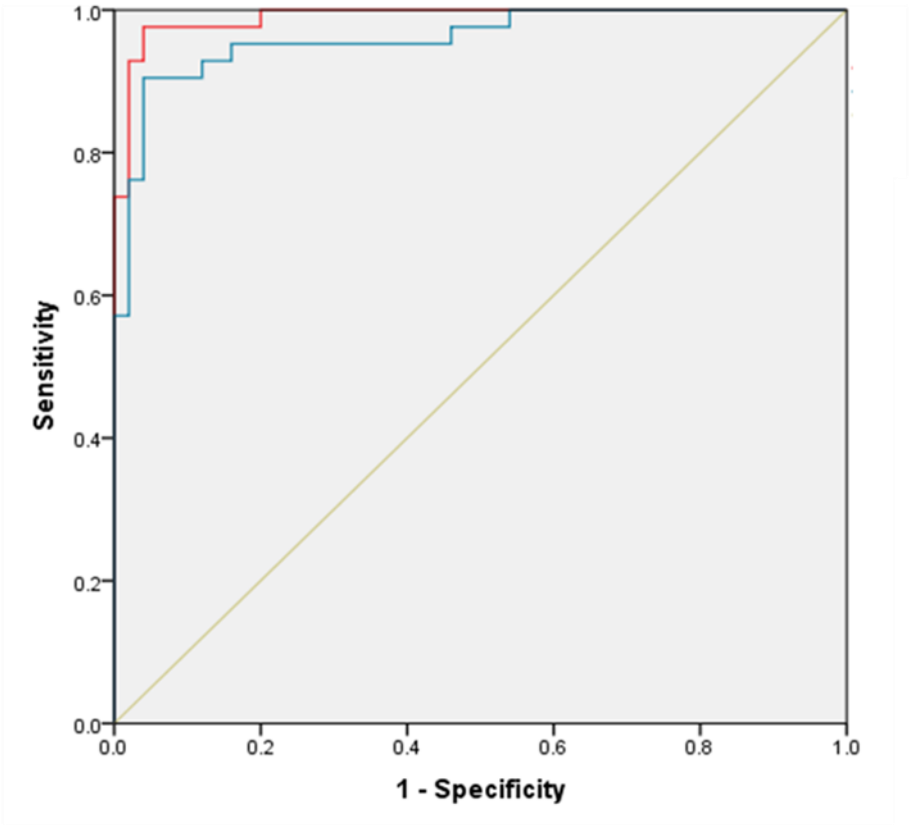
3.7 Gating strategy of BAT has important diagnostic implications

The diagnostic performance of BAT gating on SSC^{low}/CD203c+/HLA-DR- was superior to the one using SSC^{low}/CD123+/HLA-DR- with a larger area under the ROC curve (Figure 3.7). The optimal cut-off based on the ROC curve generated using the latter gating strategy resulted in a 91% diagnostic accuracy with 5% false-negatives and 3% false positives (Table 3.4). Adopting the SSC^{low}/CD203c+/HLA-DR- gating strategy resulted in a diagnostic accuracy of 97%, with 1% false-negatives and 2% false-positives[165]. Unusually, this methodological improvement resulted in both enhanced sensitivity and specificity. Figure 3.5 shows an example of a patient that would be considered false negative if gating was confined to CD123+/HLADR- cells. Furthermore, using this gating strategy, 15% of patients had less than 500 basophils in at least one condition and thus BAT would be uninterpretable.

Table 3.4 Diagnostic accuracy of the basophil activation test to peanut using different gating strategies.

The optimal cut-off was determined for the percentage of CD63-positive basophils following stimulation with 100 ng/ml of peanut extract according to the Youden index. Abbreviations: AUC ROC, area under the receiver operating characteristic curve; PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; LR-, negative likelihood ratio.

	Gating strategy	
	CD123+ HLADR-	CD203c+ HLADR-
Optimal cut-off	6.8	7.3
AUC ROC for the cut-off	0.91	0.97
Accuracy	91%	97%
Sensitivity	88%	98%
Specificity	94%	96%
PPV	93%	95%
NPV	90%	98%
%True positives	40%	45%
%False positives	3%	2%
%True negatives	51%	52%
%False negatives	5%	1%
LR+	14.7	24.4
LR-	0.13	0.02



Gating strategy	AUC	95% CI
CD203c+ CD123+ HLADR-	0.99	0.975-1.0
CD123+ HLADR-	0.96	0.922-0.998

Figure 3.7 Receiver operating characteristic curves of the average percentage of CD63-positive basophils at 10 and 100ng/ml of peanut extract using two different gating strategies to identify basophils.

SSC^{low} CD203c+ HLA-DR- (red), SSC^{low} CD123+ HLA-DR- (blue).

3.8 Conclusions

Basophils down-regulate CD123 with activation in a subset of patients and this can have significant deleterious implications in the diagnosis of allergic disease. While performing the BAT, the use of gating strategies that depend on CD123 may lead to loss-to-analysis of basophils, in particular basophils that highly express CD63 and CD203c, resulting in a false-negative outcome for the test. To overcome this limitation, the additional use of CD203c, both as an identification and activation marker, prevents the loss-to-analysis of activated basophils and allows accurate assessment of basophil activation and, consequently, a more accurate diagnosis of allergy.

Chapter 4 Basophil activation test discriminates between allergy and tolerance in peanut-sensitised children

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The basophil activation test (BAT) to peanut is an *in vitro* assay where the expression of activation markers on the surface of basophils is evaluated by flow cytometry following stimulation with peanut allergens[64, 69]. It can be seen as a surrogate of oral food challenges, where instead of feeding patients peanut *in vivo*, blood basophils are exposed to peanut *in vitro*. With a view to improve the diagnosis of peanut allergy, I sought to assess the performance of BAT to diagnose peanut allergy and to compare it to that of diagnostic tests that are currently available in clinical practice, namely skin prick test to peanut, specific IgE to peanut and specific IgE to peanut components, such as Ara h 1, Ara h 2, Ara h 3, Ara h 8 and Ara h 9.

4.1 Diagnostic study population

One hundred and nine children, 76% boys, aged from 5 months to 17 years (median 5 years), participated in this diagnostic study. Eighty-four children had suspected peanut allergy and twenty were tested only for research purposes. Patients had different pre-test probability of peanut allergy. Figure 4.1 shows the main reasons for suspecting of peanut allergy which justified the allergy testing. Sixty-six oral food challenges to peanut were performed, 52 DBPCFC and 14 open oral food challenges. Twenty challenges were positive, 41 were negative and 5 were indeterminate (3 patients refused to eat and 2 showed subjective symptoms in the absence of objective signs). These 5 patients with indeterminate outcome in the oral food challenge were excluded from the study. Four patients who passed the peanut challenge (4% of the total population and 11% of PS patients) had previously reacted to peanut or been diagnosed with peanut allergy and were considered to have outgrown peanut allergy. The study population included 104 participants, 43 peanut allergic (PA) and 61 peanut-tolerant, of which 36 were peanut-sensitised but tolerant (PS) and 25 were non-sensitised non-allergic (NA), as shown in Figure 4.2. Demographic and clinical features of the study population are represented in Table 4.1 and Figure 4.3.

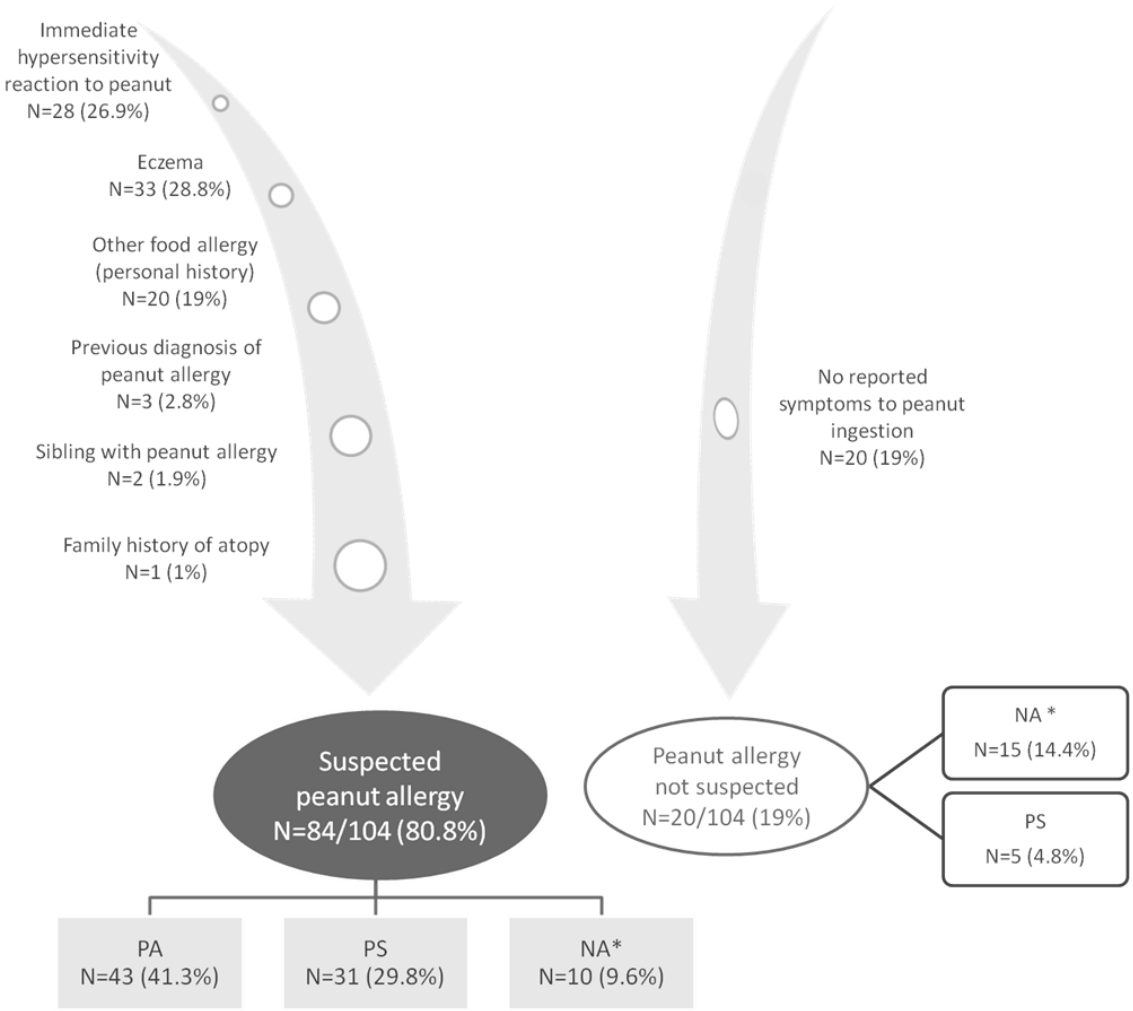

Figure 4.1 Indications for testing for peanut allergy in the study population.

*The asterisk indicates the twenty five children who were assigned to the total non-allergic (NA) group studied.
Abbreviations: PA, peanut allergic; PS, peanut sensitised but tolerant; NA, non-sensitised non-allergic to peanut.

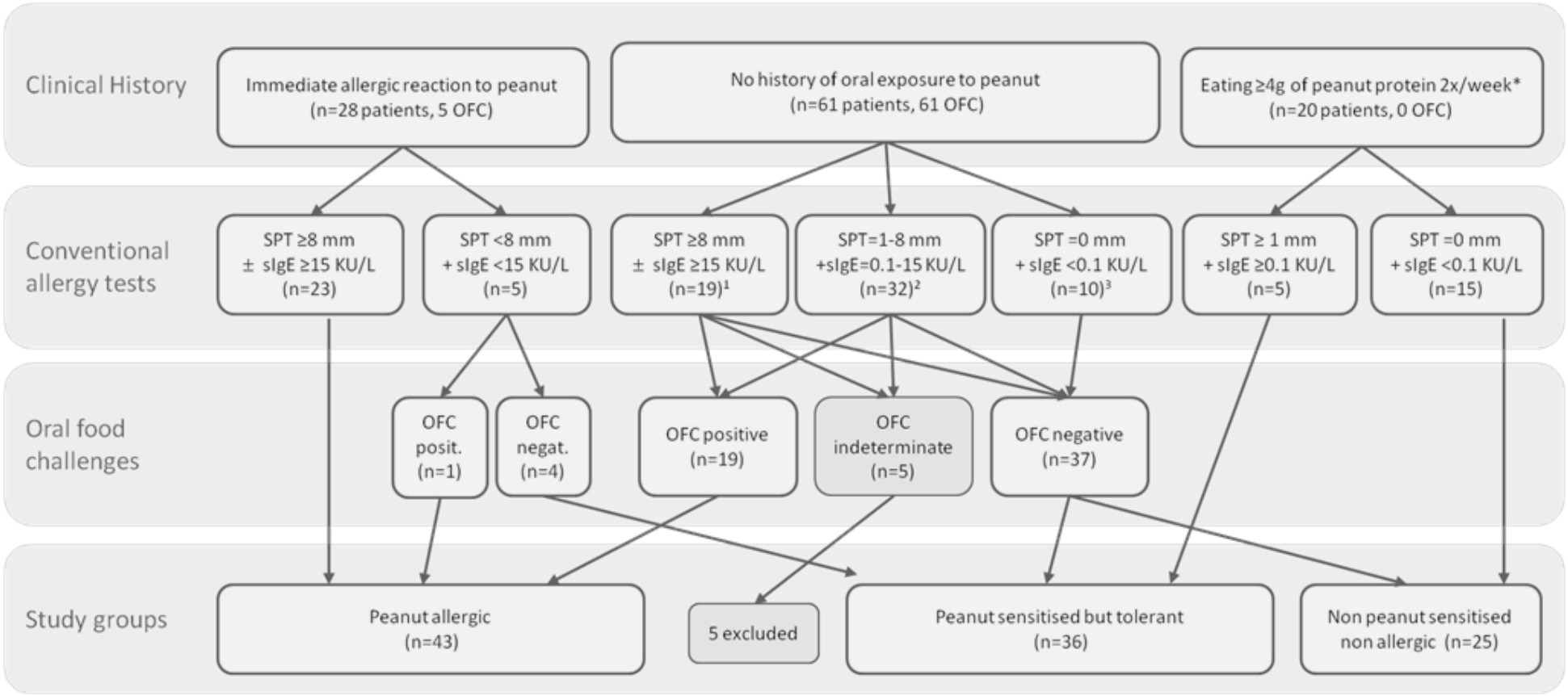

Figure 4.2 Study population.

N=104, 5 patients were excluded. *as assessed by a previously validated peanut-consumption questionnaire[17]. [1]13 positive oral food challenge, 4 negative oral food challenge, 2 indeterminate oral food challenge; [2]6 positive oral food challenge, 23 negative oral food challenge, 3 equivocal oral food challenge; [3]10 negative oral food challenge. Abbreviations: SPT, skin prick test to peanut; sIgE, specific IgE to peanut; OFC, oral food challenge to peanut; posit., positive; negat., negative.

Table 4.1 Demographic and clinical features of the primary diagnostic study population (n=104).

Values are expressed as number (percentage) or median (range).Significant p values are highlighted in bold.

Demographic and clinical characteristics	Peanut allergic (n=43)	Peanut tolerant (n=61)		p value*
		Peanut-sensitised but tolerant (n=36)	Non-peanut sensitised non-allergic (n=25)	
Age (years)	5.5 (1.5; 17.0)	4.0 (0.5;13.0)	5.0 (0.8; 13.5)	**0.005**
Males - n (%)	32 (74.4%)	23 (63.9%)	18 (72.0%)	0.366
History of oral exposure to peanut - n (%)	26 (60.5%)	7 (19.4%)	15 (60.0%)	**<0.001**
SPT to peanut (mm)	9 (2; 19)	2 (0; 12)	0 (0; 0)	**<0.001**
Specific IgE to peanut (KU_A/l)	14.50 (0.14; 604.0)	0.81 (0.01; 35.70)	0.01 (0; 0.08)	**<0.001**
Specific IgE to Ara h 1 (KU_A/l)	0.45 (0; 199.0)	0.06 (0; 3.79)	0.01 (0; 0.03)	**0.001**
Specific IgE to Ara h 2 (KU_A/l)	9.21 (0.05; 386.0)	0.06 (0.01; 1.84)	0.01 (0; 0.08)	**<0.001**
Specific IgE to Ara h 3 (KU_A/l)	0.06 (0; 89.60)	0.05 (0; 1.36)	0.01 (0; 0.04)	0.217
Specific IgE to Ara h 8 (KU_A/l)	0.08 (0; 57.80)	0.01 (0; 35.80)	0.01 (0; 0.02)	**0.027**
Specific IgE to Ara h 9 (KU_A/l)	0.01 (0; 5.62)	0.02 (0; 11.0)	0.01 (0; 0.02)	0.602
Other food allergy - n (%)	39 (90.7%)	32 (88.9%)	3 (12.0%)	1.0
Atopic eczema - n (%)	36 (83.7%)	21 (58.3%)	12 (48.0%)	**0.022**
Asthma - n (%)	13 (30.2%)	6 (16.7%)	0 (0%)	0.193
Allergic rhinitis - n (%)	14 (32.6%)	9 (25.0%)	2 (8.0%)	0.620
Pollen allergy - n (%)	14 (32.6%)	8 (22.2%)	1 (4.0%)	0.349
Non atopic - n (%)	0 (0%)	0 (0%)	12 (48.0%)	-

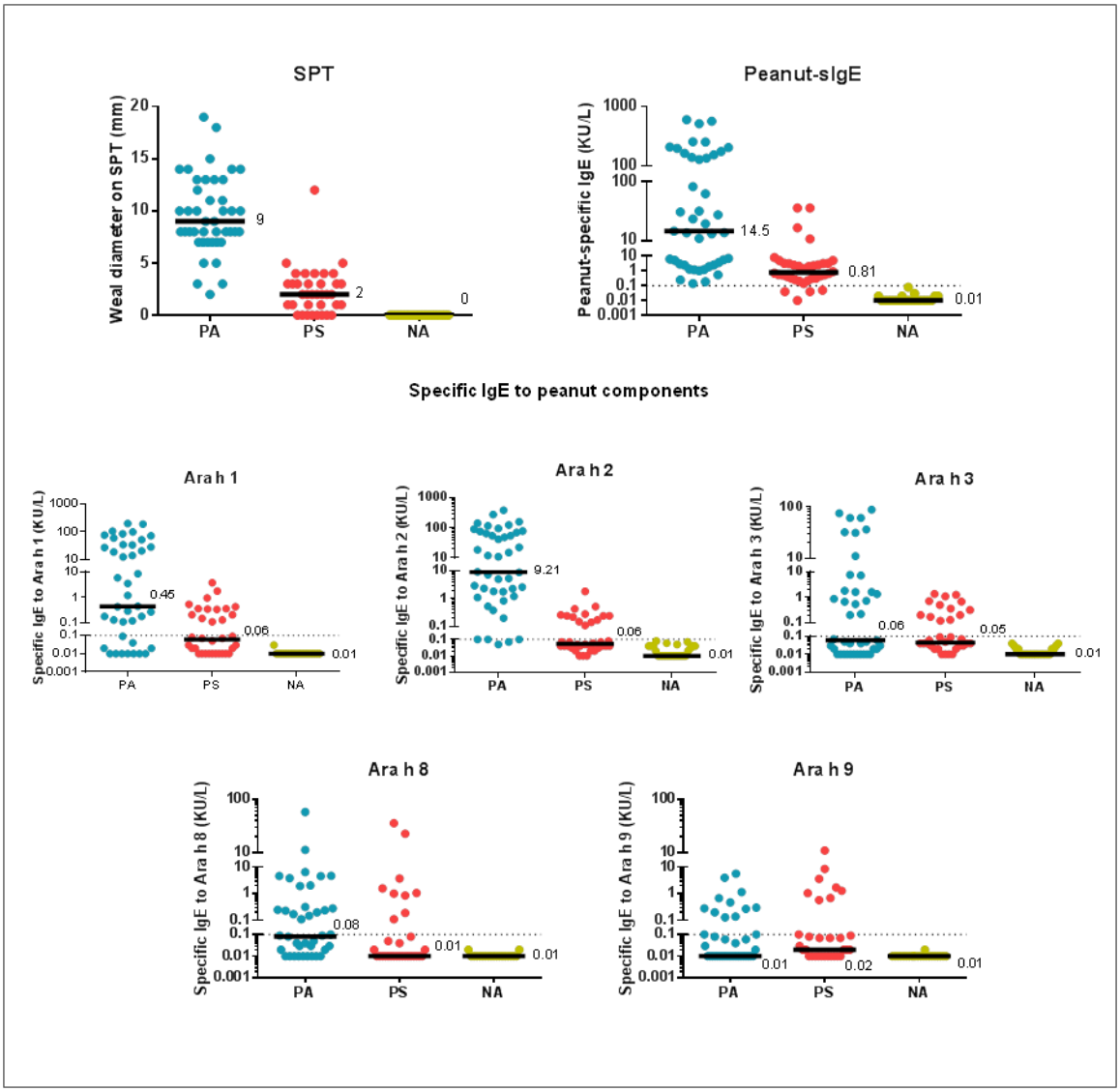

Figure 4.3 Skin prick test to peanut (SPT), specific IgE to peanut (peanut-sIgE) and specific IgE to peanut components Ara h 1, Ara h 2, Ara h 3, Ara h 8 and Ara h 9, in the three patient groups.

PA, peanut allergic; PS, peanut sensitised but tolerant; NA, non-sensitised non-allergic to peanut. The dots represent results for individual patients and the horizontal lines represent the median for each group.

4.2 Basophils of peanut allergic children show peanut dose-dependent up-regulation of activation markers

The basophils of 12 (11.5%) children were "non-responders" and were necessarily excluded from the comparison of BAT results between groups and from the ROC curve analysis; however, they were taken into account when assessing the clinical application of the BAT and its impact in the reduction of oral food challenges.

In peanut allergic children, basophils showed increased expression of CD63 and CD203c with increasing concentrations of peanut extract up to 100 ng/ml followed by a *plateau*, forming a bell-

shaped dose-response curve in the majority of cases. The basophils from peanut sensitised but tolerant children did not significantly respond to peanut ($p<0.001$ for the comparison of the median basophil activation between PA and PS patients) neither did basophils from NA children (Figure 4.4). Similar findings were observed for SI CD203c. This difference in basophil response between groups was reflected in other parameters of the basophil activation test (Table 4.2).

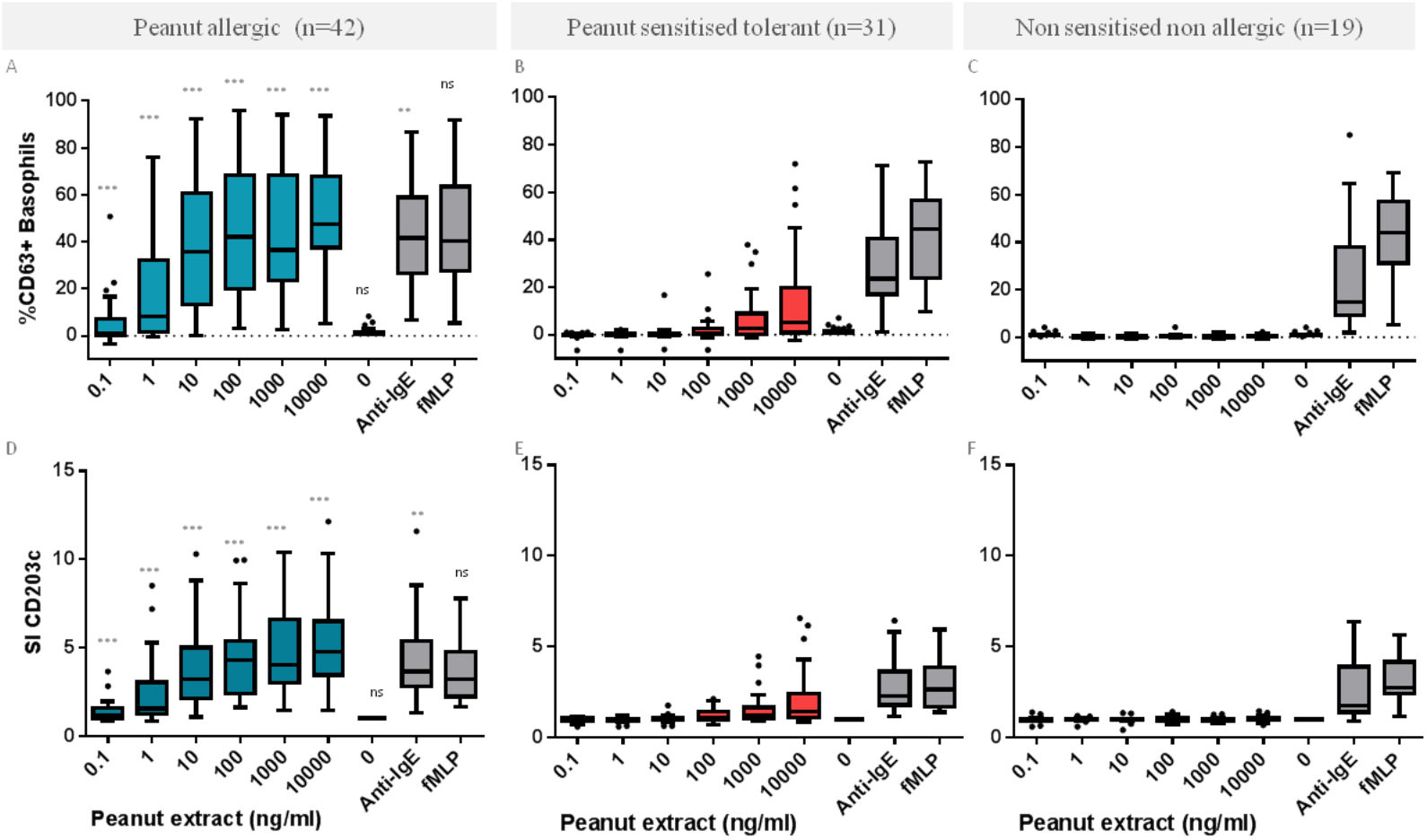

Figure 4.4 Basophil activation test to peanut in the three patient groups.

PA, peanut allergic (n=42, Fig. A and D); PS, peanut-sensitised but tolerant (n=31, Fig. B and E); NA, non-sensitised non-allergic (n=19, Fig. C and F) children. Tukey plts are represented.p value refers to the comparison between PA versus PS: ***p<0.001, **p<0.01 and ns, non-significant. 0 represents the negative control. Anti-IgE and fMLP are the positive controls.

Table 4.2 Parameters of basophil activation test (BAT) in relation to allergy versus tolerance to peanut.

N=92.Concentrations of peanut extract are expressed in ng/ml. *p values refer to the comparison between peanut allergic and peanut sensitised but tolerant patients. Abbreviations: BAT, basophil activation test; PA, peanut allergic; PS, peanut sensitised but tolerant; NA, non-sensitised non-allergic; AUC ROC, area under the receiver-operating characteristic curve; SI, stimulation index; EC_{50}, half-maximal effective concentration.

BAT to peanut	Peanut allergic (n=42)	Peanut tolerant (n=50)		p value*	AUC ROC curve (95% CI)
		PS (n=31)	NA (n=19)		
%CD63+ Peanut 0.1	0.83 (0.09, 7.18)	0.02 (0, 0.20)	0.13 (0, 0.39)	**<0.001**	0.75 (0.65, 0.86)
%CD63+ Peanut 1	8.14 (1.57, 32.05)	0 (0, 0.67)	0.23 (0, 0.64)	**<0.001**	0.92 (0.86, 0.98)
%CD63+ Peanut 10	35.88 (13.13, 60.71)	0.13 (0, 0.77)	0.25 (0.01, 0.58)	**<0.001**	0.97 (0.94, 1.0)
%CD63+ Peanut 100	42.12 (19.96, 68.45)	0.56 (0.11, 2.53)	0.36 (0, 0.84)	**<0.001**	0.99 (0.97, 1.0)
%CD63+ Peanut 1,000	36.54 (23.46, 68.5)	2.62 (0.16, 9.06)	0.49 (0, 0.74)	**<0.001**	0.96 (0.93, 1.0)
%CD63+ Peanut 10,000	47.42 (37.52, 67.79)	5.24 (0.83, 19.83)	0.41 (0.02, 0.80)	**<0.001**	0.93 (0.87, 0.98)
SI CD203c Peanut 0.1	1.14 (1.0, 1.60)	0.99 (0.93, 1.07)	0.99 (0.91, 1.04)	**<0.001**	0.75 (0.65, 0.85)
SI CD203c Peanut 1	1.56 (1.23, 3.03)	0.98 (0.93, 1.05)	1.0 (0.97, 1.03)	**<0.001**	0.93 (0.87, 0.99)
SI CD203c Peanut 10	3.23 (2.11, 5.0)	1.0 (0.96, 1.09)	1.0 (0.96, 1.03)	**<0.001**	0.98 (0.97, 1.0)
SI CD203c Peanut 100	4.27 (2.42, 5.36)	1.08 (1.0, 1.44)	1.0 (0.92, 1.08)	**<0.001**	0.99 (0.98, 1.0)
SI CD203c Peanut 1,000	4.0 (3.0, 6.6)	1.18 (1.02, 1.67)	0.97 (0.93, 1.02)	**<0.001**	0.97 (0.93, 1.0)
SI CD203c Peanut 10,000	4.78 (3.46, 6.51)	1.43 (1.1, 2.43)	1.0 (0.97, 1.09)	**<0.001**	0.93 (0.87, 0.98)
Mean CD63 Peanut 10-100	39.8 (19.7, 64.4)	0.35 (0, 1.85)	0.29 (0.4, 0.63)	**<0.001**	0.99 (0.98, 1.0)
Mean CD203c Peanut 10-100	3.69 (2.53, 5.09)	1.04 (0.98, 1.29)	1.0 (0.94, 1.05)	**<0.001**	0.99 (0.99, 1.0)

BAT to peanut	Peanut allergic (n=42)	Peanut tolerant (n=50)		p value*	AUC ROC curve (95% CI)
		PS (n=31)	NA (n=19)		
Mean CD63 Peanut 100-1,000	36.39 (25.52, 65.19)	2.04 (0.34, 6.07)	0.51 (0.10, 0.82)	**<0.001**	0.98 (0.97, 1.0)
Mean CD203c Peanut 100-1,000	4.26 (2.79, 6.29)	1.13 (1.05, 1.54)	1.0 (0.95, 1.07)	**<0.001**	0.99 (0.97, 1.0)
AUC CD63 Peanut	149.1 (88.6, 249.7)	9.2 (1.5, 21.2)	1.6 (0.5, 3.8)	**<0.001**	0.98 (0.96, 1.0)
AUC CD203c Peanut	16.9 (13.0, 22.8)	5.5 (5.1, 6.6)	5.0 (4.8, 5.3)	**<0.001**	0.99 (0.97, 1.0)
Maximal %CD63+ Peanut	59.5 (39.5, 76.8)	5.24 (1.6, 23.56)	0.84 (0.42, 1.53)	**<0.001**	0.95 (0.90, 0.99)
Maximal SI CD203c Peanut	5.5 (4.0, 7.9)	1.43 (1.2, 2.93)	1.04 (1.0, 1.12)	**<0.001**	0.95 (0.90, 0.99)
%CD63+ Peanut 100/aIgE	1.14 (0.71, 1.54)	0.02 (0.01, 0.13)	0.02 (0, 0.11)	**<0.001**	0.97 (0.95, 1.0)
%CD63+ Peanut 100/aFcεRI	1.92 (1.42, 4.49)	0.03 (0.01, 0.21)	0.02 (0, 0.11)	**<0.001**	0.96 (0.91, 1.0)[$]
EC_{50} (ng/ml) - CD63	10 (1, 10)	300 (100, 1000)[#]	-	**<0.001**	-
EC_{50} (ng/ml) - CD203c	10 (1, 10)	200 (100, 825)[+]	-	**<0.001**	-

The %CD63+ basophils in response to the negative control (p=0.958) and non-IgE-mediated positive control (fMLP, p=0.581) was similar across groups. The response to anti-IgE was higher in PA compared to PS (p=0.007). The proportion of non-responders was higher in peanut-tolerant (including peanut sensitised but tolerant and non-sensitised non-allergic children) compared to peanut allergic (11 versus 1, p=0.012) children.

4.3 Optimal diagnostic cut-off values

Peanut allergy (based on oral food challenge or 95% PPV cut-offs, n=42) and tolerance status (based on oral food challenge or peanut consumption, n=50) was the reference-point to evaluate the diagnostic performance of BAT on ROC curve analysis. The area under the ROC curve for each BAT parameter is represented in Table 4.2.

The best diagnostic cut-off values (although all cut-offs performed well without statistically significant differences between them) were obtained for the percentage of CD63-positive basophils at 100 ng/ml and for the mean percentage of CD63-positive basophils at 10 and 100 ng/ml of peanut extract (Table 4.3). These were simultaneously optimal, negative and positive decision levels, with 98% sensitivity, 96% specificity, 95% PPV, 98% NPV and 97% accuracy. The mean percentage of CD63-positive basophils at 10 and 100 ng/ml of peanut extract was the BAT parameter selected for further diagnostic analyses.

Table 4.3 Optimal cut-offs for the different parameters of basophil activation test to peanut.

The BAT parameters of choice are highlighted in bold. *LR could not be determined as sensitivity or specificity was 100%. Numbers in the left column indicate the concentration of peanut extract in ng/ml. Abbreviations: aIgE, anti-IgE; aFcεRI, anti-FcεRI; BAT, basophil activation test; AUC ROC, area under the receiver-operating characteristic curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; LR, likelihood ratio; SI, stimulation index; AUC, area under the curve. The concentrations of peanut extract in the left column are expressed in ng/ml.

BAT parameter	Cut-off (95% CI)	AUC ROC (95% CI)	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)	LR+ (95%CI)	LR- (95%CI)	Accuracy (95% CI)
%CD63+ Peanut 0.1	0.39 (0.13; 2.94)	0.73 (0.64; 0.82)	64.3% (48.0; 78.4)	82.0% (68.6; 91.4)	75.0% (57.8; 87.9)	73.2% (59.7; 84.2)	3.6 (1.9; 6.7)	0.44 (0.28; 0.67)	73.9% (64.9; 82.9)
%CD63+ Peanut 1	1.50 (0.56; 2.21)	0.88 (0.81; 0.94)	81.0% (65.9; 91.4)	94.0% (83.5; 98.7)	91.9% (78.1; 98.3)	85.5% (73.3; 93.5)	13.5 (4.5; 40.8)	0.20 (0.11; 0.38)	88.0% (81.4; 94.7)
%CD63+ Peanut 10	2.33 (2.33; 7.04)	0.96 (0.91; 1.0)	95.2% (83.8; 99.4)	96.0% (86.3; 99.5)	95.2% (83.8; 99.4)	96.0% (86.3; 99.5)	23.8 (6.1; 92.7)	0.05 (0.01; 0.19)	95.7% (91.5; 99.8)
%CD63+ Peanut 100	**8.11 (2.93; 16.47)**	**0.97 (0.93; 1.0)**	**97.6% (87.4; 99.9)**	**96.0% (86.3; 99.5)**	**95.3% (84.2; 99.4)**	**98.0% (89.1; 99.9)**	**24.4 (6.3; 95.0)**	**0.02 (0.0; 0.17)**	**96.7% (93.1; 100)**
%CD63+ Peanut 1,000	20.31 (7.35; 21.71)	0.92 (0.87; 0.98)	90.5% (77.4; 97.3)	94.0% (83.5; 98.7)	92.7% (80.1; 98.5)	92.2% (81.1; 97.8)	15.1 (5.0; 45.4)	0.10 (0.04; 0.26)	92.4% (87.0; 97.8)
%CD63+ Peanut 10,000	19.99 (9.60; 34.36)	0.88 (0.82; 0.95)	90.5% (77.4; 97.3)	86.0% (73.3; 94.2)	84.8% (70.5; 93.5)	91.5% (79.6; 97.6)	6.5 (3.2; 12.9)	0.11 (0.04; 0.28)	88.0% (81.4; 94.7)
SI CD203c Peanut 0.1	1.10 (0.99; 1.30)	0.72 (0.64; 0.81)	54.8% (38.7; 70.2)	90.0% (78.2; 96.7)	82.1% (63.1; 93.9)	70.3% (57.6; 81.1)	5.5 (2.3; 13.1)	0.50 (0.36; 0.71)	73.9% (64.9; 82.9)

SI CD203c Peanut 1	1.22 (1.06; 1.25)	0.91 (0.85; 0.97)	81.0% (65.9; 91.4)	100.0% (92.9; 100)	100.0% (89.7; 100)	86.2% (74.6; 93.9)	-*	0.19 (0.10; 0.36)	91.3% (85.5; 97.1)
SI CD203c Peanut 10	1.18 (1.18; 1.77)	0.94 (0.89; 0.99)	97.6% (87.4; 99.9_)	90.0% (78.2; 96.7)	89.1% (76.4; 96.4)	97.8% (88.5; 99.9)	9.8 (4.2; 22.5)	0.03 (0.0; 0.18)	93.5% (88.4; 98.5)
SI CD203c Peanut 100	1.88 (1.62; 2.24)	0.96 (0.91; 1.0)	95.2% (83.8; 99.4)	96.0% (86.3; 99.5)	95.2% (83.8; 99.4)	96.0% (86.3; 99.5)	23.8 (6.1; 92.7)	0.05 (0.01; 0.19)	95.7% (91.5; 99.8)
SI CD203c Peanut 1,000	1.96 (1.43; 2.62)	0.93 (0.87; 0.98)	95.2% (83.8; 99.4)	90.0% (78.2; 96.7)	88.9% (75.9; 96.3)	95.7% (85.5; 99.5)	9.5 (4.1; 21.9)	0.05 (0.03; 0.21)	92.4% (87.0; 97.8)
SI CD203c Peanut 10,000	2.68 (1.95; 3.31)	0.88 (0.82; 0.95)	90.5% (77.4; 97.3)	86.0% (73.3; 94.2)	84.4% (70.5; 93.5)	91.5% (79.6; 97.6)	6.5 (3.2; 12.9)	0.11 (0.04; 0.28)	88.0% (81.4; 94.7)
Mean CD63 Peanut 10-100	**4.78** **(4.78; 11.76)**	**0.97** **(0.93; 1.0)**	**97.6%** **(87.4; 99.9)**	**96.0%** **(86.3; 99.5)**	**95.3%** **(84.2; 99.4)**	**98.0%** **(89.1; 99.9)**	**24.4** **(6.3; 95.0)**	**0.02** **(0.0; 0.17)**	**96.7%** **(93.1; 100)**
Mean CD203c Peanut 10-100	1.40 (1.40; 1.75)	0.97 (0.94; 1.0)	100% (91.6; 100)	94.0% (83.5; 98.7)	93.3% (81.7; 98.6)	100.0% (92.5; 100)	16.7 (5.6; 49.9)	-*	96.7 % (93.1; 100)
Mean CD63 Peanut 100-1,000	19.12 (5.14; 23.29)	0.93 (0.88; 0.99)	90.5% (77.4; 97.3)	96.0% (86.3; 99.5)	95.0% (83.1; 99.4)	92.3% (81.5; 97.9)	22.6 (5.8; 88.3)	0.10 (0.04; 0.25)	93.5% (88.4; 98.5)
Mean CD203c Peanut 100-1,000	1.72 (1.72; 2.76)	0.95 (0.91; 0.99)	100% (91.6; 100)	90.0% (78.2; 96.7)	89.4% (76.9; 96.5)	100.0% (92.1; 100)	10.0 (4.4; 23.0)	-*	94.6% (89.9; 99.2)

AUC CD63 Peanut	30.01 (24.91; 84.58)	0.92 (0.87; 0.98)	92.9% (80.5; 98.5)	92.0% (80.8; 97.8)	90.7% (77.9; 97.4)	93.9% (83.1; 98.7)	11.6 (4.5; 29.8)	0.08 (0.03; 0.23)	92.4% (87.0; 97.8)
AUC CD203c Peanut	8.41 (7.31; 10.67)	0.95 (0.90; 0.99)	97.6% (87.4; 99.9)	92.0% (80.8; 97.8)	91.1% (78.8; 97.5)	97.9% (88.7; 99.9)	12.2 (4.76; 31.3)	0.03 (0; 0.18)	94.6% (89.9; 99.2)
Maximal %CD63+ Peanut	27.52 (11.22; 34.66)	0.90 (0.84; 0.96)	92.9% (80.5; 98.5)	88.0% (75.7; 95.5)	86.7% (73.2; 94.9)	93.6% (82.5; 98.7)	7.7 (3.6; 16.5)	0.08 (0.03; 0.24)	90.2% (84.1; 96.3)
Maximal SI CD203c Peanut	2.65 (2.21; 3.76)	0.90 (0.84; 0.96)	97.6% (87.4; 99.9)	82.0% (68.6; 91.4)	82.0% (68.6; 91.4)	97.6% (87.4; 99.9)	5.42 (3.0; 9.8)	0.03 (0.0; 0.20)	89.1% (82.8; 95.5)
%CD63+ Peanut 100/aIgE	0.52 (0.12; 0.62)	0.92 (0.86; 0.98)	88.1% (74.4; 96.0)	96.0% (86.3; 99.5)	94.9% (82.7; 99.4)	90.6% (79.3; 96.9)	22.0 (5.6; 86.0)	0.12 (0.05; 0.28)	92.4% (87.0; 97.8)
%CD63+ Peanut 100/aFcεRI	0.37 (0.37; 1.32)	0.94 (0.89; 0.99)	97.2% (85.5; 99.9)	91.1% (78.8; 97.5)	89.7% (75.8; 97.1)	97.6% (87.4; 99.9)	10.9 (4.3; 27.9)	0.03 (0.0; 0.21)	93.8% (88.6; 99.1)

The area under the ROC curve for BAT (considering the mean CD63 10-100) was superior to that for other allergy tests, namely for SPT to peanut and specific IgE to peanut and to Ara h 1, Ara h 2, Ara h 3, Ara h 8 and Ara h 9 (Table 4.4, Figure 4.5). Ara h 2-specific IgE performed better than specific IgE to the other peanut components.

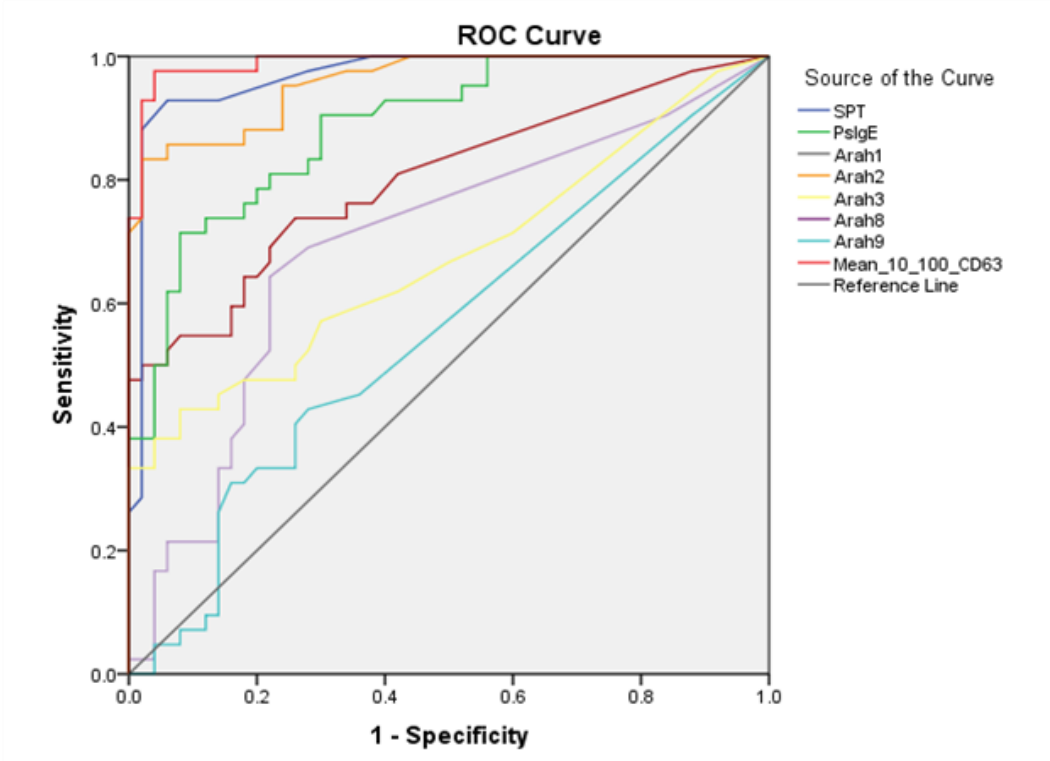

Figure 4.5 Receiver operating characteristic curves

Receiver operating characteristic curves for the basophil activation test (mean CD63 10-100, red), skin prick test (dark blue), specific IgE to peanut (green) and specific IgE to Ara h 1 (violet), to Ara h 2 (orange), to Ara h 3 (yellow), to Ara h 8 (maroon) and to Ara h 9 (light blue).

Table 4.4 Performance of different tests in the diagnosis of peanut allergy.

For the basophil activation test, the average percentage of CD63-positive basophils at 10 and 100 ng/ml of peanut extract (mean CD63 10-100) was considered. Non-responders were excluded as there was no outcome for BAT. Abbreviations: AUC ROC, area under the receiver operating characteristic curve; 95% CI, 95% confidence interval; PPV, positive predictive value; NPV, negative predictive value; LR, likelihood ratio; SPT, skin prick test to peanut; BAT, basophil activation test.

Diagnostic tests	Optimal cut-off	AUC ROC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	LR+ (95% CI)	LR- (95% CI)	Diagnostic accuracy (95% CI)
SPT (mm)	5 (5; 7)	0.93 (0.88; 0.99)	92.9% (80.5; 98.5)	94.0% (83.5; 98.7)	92.9% (80.5; 98.5)	94.0% (83.5; 98.7)	15.5 (5.15; 46.5)	0.08 (0.03; 0.23)	93.5% (88.4; 98.5)
Specific IgE to peanut (KU_A/l)	5.35 (1.04; 10.9)	0.81 (0.73; 0.89)	69.0% (52.9; 82.4)	92.0% (80.8; 97.8)	87.9% (71.8; 96.6)	78.0% (65.3; 87.7)	8.63 (3.3; 22.6)	0.34 (0.21; 0.53)	81.5% (73.6; 89.5)
Ara h 2-specific IgE (KU_A/l)	0.53 (0.10; 2.29)	0.91 (0.85; 0.97)	83.3% (68.6; 93.0)	98.0% (89.4; 99.9)	97.2% (85.5; 99.9)	87.5 % (75.9; 94.8)	41.7 (5.96; 291)	0.17 (0.09; 0.34)	91.3% (85.5; 97.1)
BAT	4.78 (4.78; 11.76)	0.97 (0.93; 1.0)	97.6% (87.4; 99.9)	96.0% (86.3; 99.5)	95.3% (84.2; 99.4)	98.0% (89.1; 99.9)	24.4 (6.3; 95.0)	0.02 (0.0; 0.17)	96.7% (93.1; 100)

4.4 Validation of diagnostic cut-off values in an independent population

In order to externally validate my findings, 65 children (25 peanut allergic, 24 peanut-sensitised but tolerant and 16 non-sensitised non-allergic) were prospectively recruited from an independent population. Demographic and clinical characteristics of this validation population are represented in Table 4.5. They underwent the same study procedures as the primary study population. The majority (94%) of these children underwent oral food challenge. All positive oral food challenges were DBPCFC.

Table 4.5 Demographic and clinical characteristics of the external validation population

N=65. Values are expressed as number (percentage) or median (range). *p value refers to the comparison between peanut allergic and peanut sensitised but tolerant patients and significant values are highlighted in bold.

Demographic and clinical characteristics	Peanut allergic (n=25)	Peanut tolerant (n=40)		p value*
		Peanut-sensitised but tolerant (n=24)	Non-peanut sensitised non-allergic (n=16)	
Age (years)	5.3 (1.7; 13.2)	6.0 (0.5; 15.8)	5.2 (4.8; 7.0)	0.689
Males - n (%)	20 (80.0%)	14 (58.3%)	10 (62.5%)	0.128
Oral exposure to peanut - n (%)	8 (32.0%)	8 (33.3%)	9 (56.3%)	1.0
SPT to peanut (mm)	9 (1; 17)	4 (0; 9)	0 (0; 0)	**<0.001**
Specific IgE to peanut (KU_A/l)	6.13 (0.15; 194.0)	1.53 (0.07; 22.20)	0.01 (0.01; 0.08)	**0.021**
Specific IgE to Ara h 1 (KU_A/l)	0.21 (0.01; 74.70)	0.08 (0.01; 11.70)	0.01 (0.01; 0.05)	0.128
Specific IgE to Ara h 2 (KU_A/l)	1.65 (0.01; 142.0)	0.12 (0; 7.0)	0.01 (0.01; 0.01)	**<0.001**
Specific IgE to Ara h 3 (KU_A/l)	0.04 (0.01; 15.20)	0.06 (0.01; 7.28)	0.01 (0.01; 0.05)	0.864
Specific IgE to Ara h 8 (KU_A/l)	0.03 (0.01; 9.80)	0.04 (0.01; 62.30)	0.01 (0.01; 0.17)	0.980
Specific IgE to Ara h 9 (KU_A/l)	0.01 (0.01; 7.0)	0.04 (0.01; 11.90)	0.01 (0.01; 0.01)	0.084
Other food allergy - n (%)	24 (96.0%)	21 (87.5%)	6 (37.5%)	0.349
Atopic eczema - n (%)	20 (80.0%)	18 (75.0%)	14 (87.5%)	0.742
Asthma - n (%)	14 (56.0%)	6 (25.0%)	7 (43.8%)	**0.042**
Allergic rhinitis - n (%)	19 (76.0%)	11 (45.8%)	10 (62.5%)	**0.042**
Pollen allergy - n (%)	12 (48.0%)	11 (45.8%)	7 (43.8%)	1.0
Non atopic - n (%)	0 (0%)	0 (0%)	2 (12.5%)	-

Four (6%) patients had non-responder basophils and were excluded from further analyses. Applying the optimal cut-off previously determined for the average percentage of CD63-positive basophils at 10 and 100ng/ml of peanut extract, BAT showed 100% specificity, 83.3% sensitivity, 100% PPV, 90.2% NPV and 93.4% accuracy. BAT proved to be superior to SPT to peanut, specific IgE to peanut and to Ara h 2 (Table 4.6), as was observed in the primary study population.

Table 4.6 External validation of diagnostic cut-offs of BAT in comparison with other allergy tests.

N=61, non-responders were excluded. The following optimal cut-offs were applied: for BAT, Mean CD63 10-100 ≥4.78% ; for SPT, ≥5 mm; for peanut-specific IgE, ≥5.35 KU_A/l; and for Ara h 2-specific IgE, ≥0.53 KU_A/l. Abbreviations: BAT, basophil activation test; SPT, skin prick test to peanut; 95% CI, 95% confidence interval; PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; LR-, negative likelihood ratio; *LR+ could not be determined as specificity was 100%.

Diagnostic tests	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	LR+ (95% CI)	LR- (95% CI)	Diagnostic accuracy (95% CI)
SPT	83.3% (74.0; 92.7)	83.8% (74.5; 93.0)	76.9% (66.3; 87.5)	88.6% (80.6; 96.6)	5.14 (2.91; 13.31)	0.20 (0.1; 0.3)	83.6% (74.3; 92.9)
Specific IgE to peanut	50.0% (37.5; 62.5)	83.8% (74.5; 93.0)	66.7% (54.8; 78.5)	72.1% (60.8; 83.3)	3.08 (1.47; 8.98)	0.60 (0.5; 0.7)	70.5% (59.0; 81.9)
Ara h 2-specific IgE	65.0% (53.0; 77.0)	97.2% (93.1; 100.0)	92.9% (86.4; 99.3)	83.3% (74.0; 92.7)	23.4 (7.68; -*)	0.36 (0.2; 0.5)	85.7% (76.9; 94.5)
BAT	83.3% (74.0; 92.7)	100.0% (100.0; 100.0)	100.0% (100.0; 100.0)	90.2% (82.8; 97.7)	-*	0.17 (0.07; 0.26)	93.4% (87.2; 99.7)

4.5 The use of the basophil activation test in peanut-sensitised children with equivocal diagnosis

Considering the practical aspects involved in the performance of BAT (namely the use of fresh blood and the performance of cell stimulation and flow cytometry on the same day of blood collection), it did not seem feasible to apply this test to all patients with suspected peanut allergy. Apart from the feasibility, it would probably not offer additional value to the allergy tests currently used in clinical practice, such as SPT and specific IgE, which are informative in some cases. Therefore, I hypothesised that BAT was most useful in cases where the results of SPT and/or specific IgE fell in the immunological grey area (Figure 1.6) or, when considered together with the clinical history, were inconclusive. The utility of BAT was further assessed in the subgroup (n=44) of the primary study population with equivocal history and inconclusive results of SPT, peanut-specific IgE and CRD. Table 4.7 represents the demographic and clinical features of this subpopulation.

Table 4.7 Demographic and clinical features of subgroup of the primary study population with equivocal clinical history and inconclusive skin prick test and specific IgE results

N=44. Values are expressed as number (percentage) or median (range). Significant p values are highlighted in bold.

Demographic and clinical characteristics	Peanut allergic (n=8)	Peanut-sensitised but tolerant (n=36)	p value
Age (years)	5.0 (2.0; 6.0)	4.0 (0.5; 13.0)	0.964
Males - n (%)	4 (50.0%)	23 (63.9%)	0.690
History of oral exposure to peanut - n (%)	0 (0%)	7 (19.5%)	0.618
SPT to peanut (mm)	7 (2; 9)	2 (0; 12)	**0.002**
Specific IgE to peanut (KU_A/l)	0.94 (0.14; 14.50)	0.81 (0.01; 35.70)	0.964
Specific IgE to Ara h 1 (KU_A/l)	0.03 (0.01; 8.67)	0.06 (0; 3.79)	0.622
Specific IgE to Ara h 2 (KU_A/l)	0.15 (0.05; 8.95)	0.06 (0.01; 1.84)	**0.023**
Specific IgE to Ara h 3 (KU_A/l)	0.01 (0.01; 1.62)	0.05 (0; 1.36)	0.189
Specific IgE to Ara h 8 (KU_A/l)	0.01 (0.01; 4.66)	0.01 (0; 35.80)	0.893
Specific IgE to Ara h 9 (KU_A/l)	0.01 (0.01; 0.28)	0.02 (0; 11.0)	0.823
Other food allergy - n (%)	8 (100%)	32 (88.9%)	1.0
Atopic eczema - n (%)	5 (62.5%)	21 (58.3%)	1.0
Asthma - n (%)	0 (0%)	6 (16.7%)	0.573
Allergic rhinitis - n (%)	0 (0%)	9 (25%)	0.175
Pollen allergy - n (%)	0 (0%)	8 (22.2%)	0.284
Non atopic - n (%)	0 (0%)	0 (0%)	-

Three Paediatric Allergy consultants were asked to classify these 44 patients as peanut allergic or peanut tolerant based on the clinical history, SPT and specific IgE to peanut and to peanut components. In the majority of cases (46-64%) the physicians could not decide about the allergic status to peanut without doing an oral food challenge. They correctly diagnosed 26-36% and misclassified 9-16% of cases, of which 14% were false-negatives. The agreement between the three pairs of physicians was poor to fair with κ values of 0.16, 0.29 and 0.36. The 3 specialists agreed in 16 (36%) cases: 4 (9%) correctly diagnosed, 1 (2%) misclassified and 11 (25%) where they were unable to decide. In contrast, BAT provided 36 (82%) correct diagnoses, 2 (5%) false-

positives, 1 (2%) false-negative and required 5 (11%) oral food challenges. The performance of the various allergy tests was assessed by ROC curve analyses (Figure 4.6 and Table 4.8). Excluding non-responders and considering optimal cut-offs, BAT had a diagnostic accuracy of 95% (Table 4.9). This is in contrast with specific IgE to peanut, Ara h 2-specific IgE and SPT, whose accuracy suffered in this subgroup.

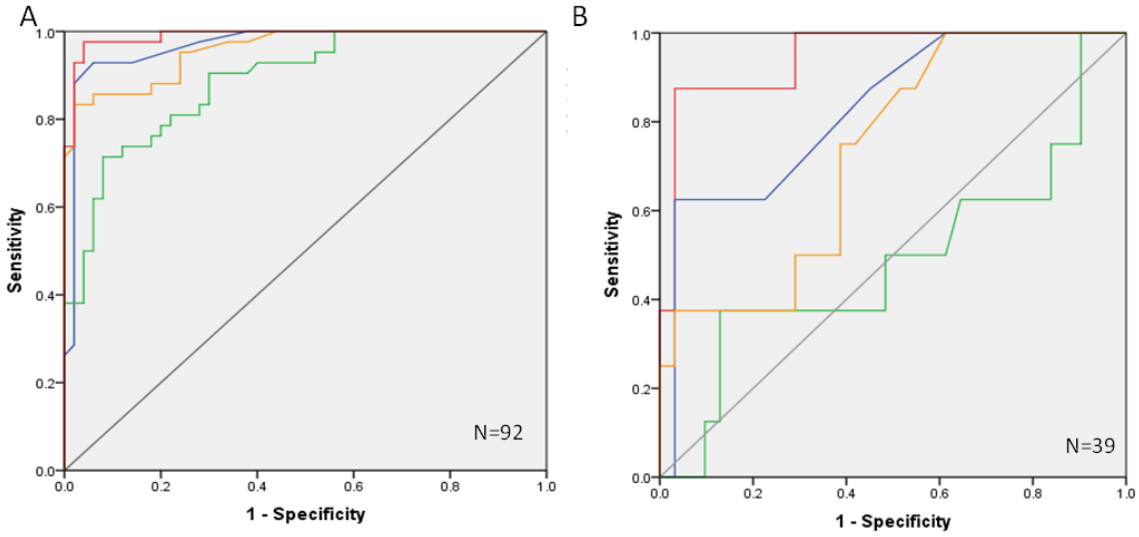

Figure 4.6 Receiver operating characteristic curves

Receiver operating characteristic curves for basophil activation test (red), skin prick test (blue), specific IgE to peanut (green) and specific IgE to Ara h 2 (orange) for (A) the whole study population (n=92) and (B) children with equivocal history, SPT and specific IgE to peanut and its components (n=39).

Table 4.8 Area under the receiver operating characteristic curve for the different allergy tests considering the whole study population and the subgroup with equivocal diagnosis.

Abbreviations: AUC ROC, area under the receiver operating characteristic curve; 95% CI, 95% confidence interval; SPT, skin prick test to peanut; BAT, basophil activation test.

Allergy tests	AUC ROC (95% CI)	
	Whole population (n=92)	Equivocal population (n=39)
SPT	0.97 (0.93, 1.0)	0.83 (0.67, 0.98)
Specific IgE to peanut	0.89 (0.82, 0.95)	0.49 (0.24, 0.74)
Ara h 2-specific IgE	0.96 (0.92, 0.99)	0.73 (0.55, 0.91)
BAT	0.99 (0.98, 1.0)	0.95 (0.87, 1.0

Table 4.9 Performance of different tests in the diagnosis of peanut allergy in the clinically equivocal population.

N=39, non-responders were excluded as there was no outcome for BAT. For BAT, the average percentage of CD63-positive basophils at 10 and 100ng/ml of peanut extract was considered. *LR- could not be determined because sensitivity was 100%. Abbreviations: AUC ROC, area under the receiver operator characteristic curve; PPV, positive predictive value; NPV, negative predictive value; LR, likelihood ratio; 95% CI, 95% confidence interval; SPT, skin prick test to peanut; BAT, basophil activation test.

Diagnostic tests	Optimal cut-off	AUC ROC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	LR+ (95% CI)	LR- (95% CI)	Diagnostic accuracy (95% CI)
SPT	7 (2; 7)	0.80 (0.61; 0.98)	62.5% (24.5; 91.5)	96.8% (83.3; 99.9)	83.3% (35.9; 99.6)	90.9% (75.7; 98.1)	19.4 (2.6; 143.0)	0.39 (0.16; 0.95)	89.7% (80.2; 99.3)
Specific IgE to peanut	0.25 (0.19; 14.5)	0.46 (0.28; 0.63)	75.0 (34.9; 96.8)	16.1% (5.5; 33.7)	18.8% (7.2; 36.4)	71.4 % (29.0; 96.3)	0.9 (0.6; 1.4)	1.55 (0.37; 6.57)	28.2% (14.1; 42.3)
Ara h 2-specific IgE	0.05 (0.05; 5.05)	0.69 (0.61; 0.78)	100.0 (63.1; 100)	38.7% (21.8; 57.8)	29.6% (13.8; 50.2)	100% (73.5; 100)	1.6 (1.2; 2.2)	-*	51.3% (35.6; 67.0)
BAT	11.75 (1.43; 17.63)	0.92 (0.80; 1.0)	87.5 (47.3; 99.7)	96.8 % (83.3; 99.9)	87.5% (47.3; 99.7)	96.8 % (83.3; 99.9)	27.1 (3.9; 190.0)	0.13 (0.02; 0.81)	95.0% (87.9; 100)

4.6 The combination of basophil activation test with other diagnostic tests

The diagnostic performance of different tests was evaluated in the primary study population (n=104), including BAT non-responders, in three ways (Table 4.10):

1. Considering each test on its own;

2. Considering the results of different diagnostic tests simultaneously; and

3. Considering BAT as a second or third sequential step in the diagnostic process, performed in patients where the results of single or combinations of standard allergy tests were equivocal.

Table 4.10 Performance of allergy tests in the diagnosis of peanut allergy.

N=104. Results are presented as number of patients (% of total study population). *The proportion of correct diagnoses was determined as ("true-positives"+"true-negatives")/n=104.[‡]For BAT, excluding non-responders, the proportion of correct diagnoses was 96.7%.**Reduction in oral food challenges was calculated in comparison with the number of oral food challenges following SPT and specific IgE (i.e. 36 oral food challenges); negative numbers represent a decrease and positive numbers an increase in the number of oral food challenges required. ***For BAT, 4.78% for the average of CD63-positive basophils at 10 and 100ng/ml of peanut extract was used as the diagnostic cut-off point. Abbreviations: SPT, skin prick test to peanut; Specific IgE, peanut-specific IgE; Ara h 2, specific IgE to Ara h 2; BAT, basophil activation test; OFC, oral food challenges.

Single diagnostic tests	Correct diagnoses*	Number of false positives	Number of false negatives	Number of BAT	Number of OFC	Change in number of OFC**
SPT	78 (75%)	1 (1%)	1 (1%)	-	24 (23%)	-12 (-33%)
Specific IgE	57 (55%)	3 (3%)	3 (3%)	-	41 (39%)	+5 (+13%)
Ara h 2	82 (79%)	1 (1%)	2 (2%)	-	19 (18%)	-17 (-46%)
BAT***	89 (86%[‡])	2 (2%)	1 (1%)	104 (100%)	12 (12%)	-24 (-67%)
Combination of diagnostic tests	**Correct diagnoses***	**Number of false positives**	**Number of false negatives**	**Number of BAT**	**Number of OFC**	**Change in number of OFC****
SPT + Specific IgE	67 (64%)	1 (1%)	0 (0%)	-	36 (35%)	0 (0%)
Specific IgE + Ara h 2	66 (63%)	1 (1%)	1 (1%)	-	36 (35%)	0 (0%)
Specific IgE + BAT	66 (63%)	1 (1%)	2 (2%)	104 (100%)	35(34%)	-1 (-3%)
SPT + BAT	77 (74%)	2 (2%)	0 (0%)	104 (100%)	25 (25%)	-11 (-31%)
SPT + Ara h 2	78 (75%)	1 (1%)	0 (0%)	-	25 (24%)	-11 (-31%)

Ara h 2 + BAT	77 (74%)	1 (1%)	2 (2%)	104 (100%)	24 (24%)	-12 (-33%)
SPT + Specific IgE + Ara h 2	67(64%)	1 (1%)	0 (0%)	-	36 (35%)	0 (0%)
SPT + Ara h 2 + BAT	70 (67%)	1 (1%)	0 (0%)	104 (100%)	33 (33%)	-3 (-8%)
SPT + Specific IgE + BAT	63 (61%)	2 (2%)	0 (0%)	104 (100%)	39 (38%)	+3 (+8%)
Specific IgE + Ara h 2 + BAT	63 (61%)	1 (1%)	1 (1%)	104 (100%)	39 (38%)	+3 (+8%)
SPT + Specific IgE + Ara h 2 + BAT	60 (58%)	1 (1%)	0 (0%)	104 (100%)	43 (42%)	+7 (+19%)
BAT as a second step in the diagnostic process	Correct Diagnoses*	Number of false positives	Number of false negatives	Number of BAT	Number of OFC	Change in number of OFC**
SPT → BAT	98 (94%)	3 (3%)	2 (2%)	24 (23%)	1 (1%)	-35 (-97%)
Specific IgE → BAT	93 (89%)	5 (5%)	3 (3%)	41 (39%)	3 (3%)	-33 (-92%)
Ara h 2 → BAT	99 (95%)	2 (2%)	2 (2%)	19 (18%)	1 (1%)	-35 (-97%)
SPT + Specific IgE → BAT	97 (93%)	3(3%)	1 (1%)	36 (35%)	3 (3%)	-33 (-92%)
SPT + Ara h 2 → BAT	99 (95%)	2 (2%)	1 (1%)	38 (37%)	2 (2%)	-34 (-94%)
SPT + Specific IgE + Ara h 2 → BAT	96 (92%)	3 (3%)	1 (1%)	36 (35%)	4 (4%)	-32 (-89%)
BAT as a third step in the diagnostic process	Correct Diagnoses*	Number of false positives	Number of false negatives	Number of BAT	Number of OFC	Change in number of OFC**
SPT → Ara h 2 → BAT	98 (94%)	3(3%)	3(3%)	6 (6%)	0 (0%)	-36 (-100%)

Considering single tests, BAT performed best and allowed a reduction in the number of oral food challenges by two-thirds, followed by Ara h 2-specific IgE and SPT to peanut. Peanut-specific IgE on its own performed the poorest, conferring the highest number of oral food challenges and correctly diagnosing only 55% of patients. Considering combinations of allergy tests, it was best to combine two different tests as opposed to three or four tests, which led to an increase in the equivocal cases because tests gave contradictory results with consequent increase in the number of oral food challenges. All combinations of tests required an increase between 2 and 3.5-fold in the number of oral food challenges compared to BAT alone.

With a view to apply BAT in clinical practice, the role of BAT as a second or third step in the diagnostic work-up was assessed, which would require a smaller number of BAT and would probably prove most useful, as shown in Section 4.5 (Table 4.10, Figure 4.7). The 2-step strategy significantly reduced the number of oral food challenges, more than using Ara h 2-specific IgE as a second step to SPT or to peanut-specific IgE (Table 4.11.), as proposed by Dang et al[44]. The 3-step sequential strategy of SPT→Ara h 2-specific IgE→BAT (Table 4.10, Figure 4.7) further reduced the number of oral food challenges to zero at the expense of a slightly higher number of false-negative results (n=3).

Table 4.11 Performance of Ara h 2-specific IgE as a second step in the diagnostic process, following SPT or specific IgE to peanut.

N=104. Results are presented as number of patients (% of total study population). *The proportion of correct diagnoses was determined as ("true-positives"+"true-negatives")/n=104. **Reduction in oral food challenges was calculated in comparison with the number of oral food challenges following SPT and specific IgE (i.e. 36 oral food challenges); negative numbers represent a decrease in the number of oral food challenges required compared to using SPT and specific IgE.

Ara h 2-specific IgE as a second step in the diagnostic process	Correct diagnoses*	False positives	False negatives	Change in number of oral food challenges**
SPT → Ara h 2	93 (89%)	2 (2%)	3 (3%)	-30 (-83%)
Specific IgE → Ara h 2	84 (81%)	3 (3%)	4 (4%)	-23 (-64%)

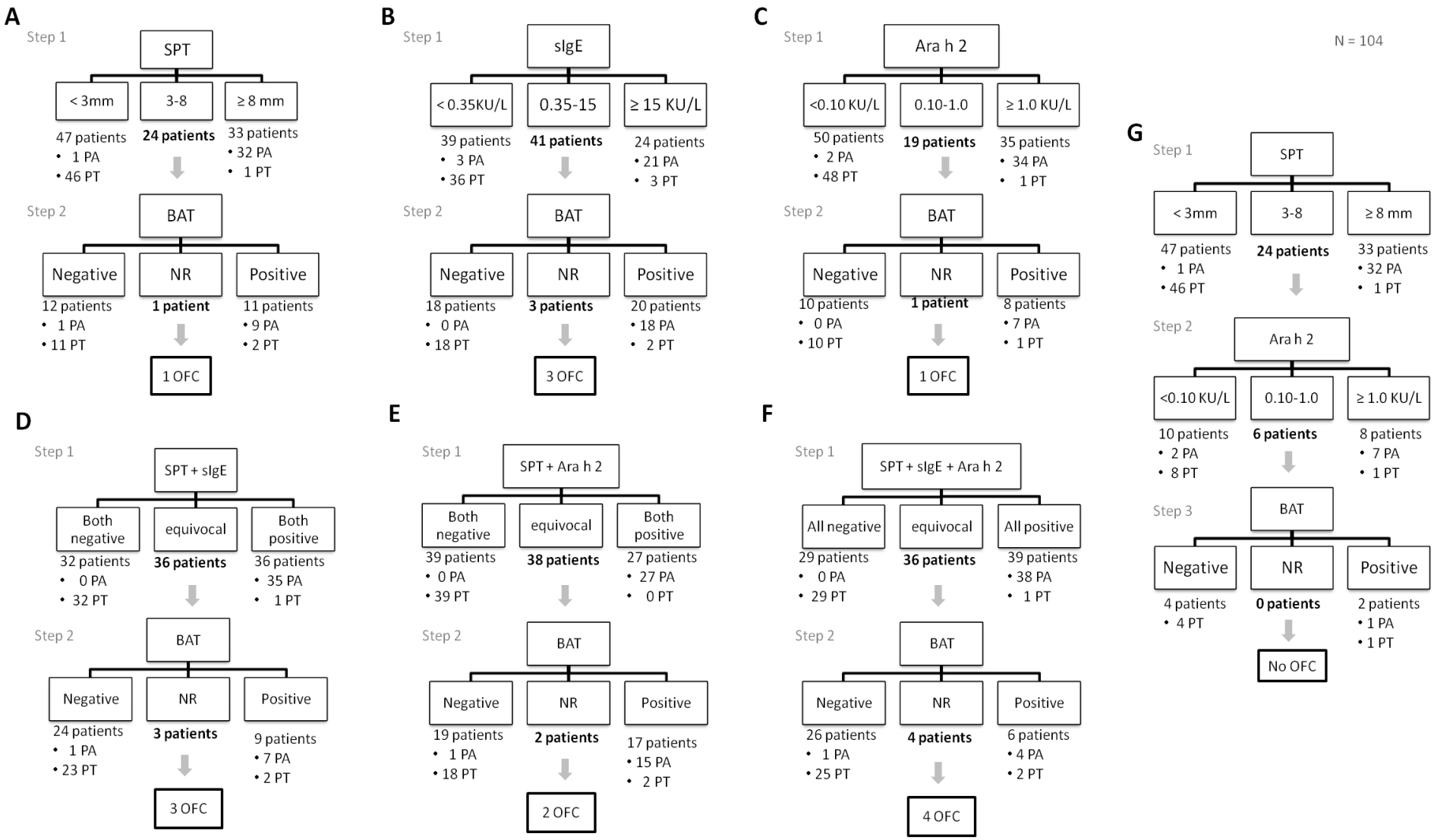
A
Step 1
SPT
< 3mm
3-8
≥ 8 mm
47 patients
• 1 PA
• 46 PT
24 patients
33 patients
• 32 PA
• 1 PT
Step 2
BAT
Negative
NR
Positive
12 patients
• 1 PA
• 11 PT
1 patient
11 patients
• 9 PA
• 2 PT
1 OFC
B
Step 1
sIgE
< 0.35KU/L
0.35-15
≥ 15 KU/L
39 patients
• 3 PA
• 36 PT
41 patients
24 patients
• 21 PA
• 3 PT
Step 2
BAT
Negative
NR
Positive
18 patients
• 0 PA
• 18 PT
3 patients
20 patients
• 18 PA
• 2 PT
3 OFC
C
Step 1
Ara h 2
<0.10 KU/L
0.10-1.0
≥ 1.0 KU/L
50 patients
• 2 PA
• 48 PT
19 patients
35 patients
• 34 PA
• 1 PT
Step 2
BAT
Negative
NR
Positive
10 patients
• 0 PA
• 10 PT
1 patient
8 patients
• 7 PA
• 1 PT
1 OFC
N = 104
G
Step 1
SPT
< 3mm
3-8
≥ 8 mm
47 patients
• 1 PA
• 46 PT
24 patients
33 patients
• 32 PA
• 1 PT
Step 2
Ara h 2
<0.10 KU/L
0.10-1.0
≥ 1.0 KU/L
10 patients
• 2 PA
• 8 PT
6 patients
8 patients
• 7 PA
• 1 PT
Step 3
BAT
Negative
NR
Positive
4 patients
• 4 PT
0 patients
2 patients
• 1 PA
• 1 PT
No OFC
D
Step 1
SPT + sIgE
Both negative
equivocal
Both positive
32 patients
• 0 PA
• 32 PT
36 patients
36 patients
• 35 PA
• 1 PT
Step 2
BAT
Negative
NR
Positive
24 patients
• 1 PA
• 23 PT
3 patients
9 patients
• 7 PA
• 2 PT
3 OFC
E
Step 1
SPT + Ara h 2
Both negative
equivocal
Both positive
39 patients
• 0 PA
• 39 PT
38 patients
27 patients
• 27 PA
• 0 PT
Step 2
BAT
Negative
NR
Positive
19 patients
• 1 PA
• 18 PT
2 patients
17 patients
• 15 PA
• 2 PT
2 OFC
F
Step 1
SPT + sIgE + Ara h 2
All negative
equivocal
All positive
29 patients
• 0 PA
• 29 PT
36 patients
39 patients
• 38 PA
• 1 PT
Step 2
BAT
Negative
NR
Positive
26 patients
• 1 PA
• 25 PT
4 patients
6 patients
• 4 PA
• 2 PT
4 OFC

Figure 4.7 Diagnosing peanut allergy using BAT sequentially in the cases where SPT, specific IgE to peanut and/or Ara h 2-specific IgE were equivocal.

Equivocal cases are marked in bold). In this simulation, oral food challenges were done in cases where BAT was indeterminate (i.e. “non-responders”). A-C: 2-step diagnostic strategy where BAT follows single tests; D-F, 2-step diagnostic strategy where BAT follows combinations of tests; G, 3-step diagnostic strategy. Abbreviations: SPT, skin prick test; sIgE, specific IgE; Ara h 2, specific IgE to Ara h 2; NPV, negative predictive value; PPV, positive predictive value; PA, peanut allergic; PS, peanut sensitised but tolerant; NA, non-peanut-sensitised non-allergic; BAT, basophil activation test; NR, non-responders; OFC, oral food challenges.

4.7 Conclusions

Considering SPT, peanut-specific IgE, specific IgE to peanut components and BAT, BAT has the best diagnostic profile. Combinations of tests offer no significant advantage to BAT alone and lead to an increase in the number of oral food challenges. The most accurate and cost-effective analysis appears to be that of using a 2-step sequential approach where SPT or Ara h 2-specific IgE is followed by BAT in the equivocal cases. In order to maximize safety and decrease false-negative tests to 0%, the 2-step sequential approach can be modified to do oral food challenges in the cases with equivocal BAT as well as in the BAT-negative patients. One should bear in mind the limitations of oral food challenges (3% false-negative[120], 3% false-positive[125] and 2-9% indeterminate outcomes[121, 122]). Future studies will determine whether BAT can add to the oral food challenge as an *in vitro* gold-standard.

Chapter 5 Distinct parameters of the basophil activation test reflect the threshold and the severity of allergic reactions to peanut

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Currently, there are no reliable objective biomarkers to predict the severity of future allergic reactions or the threshold dose at which an individual peanut allergic patient may react. Such markers would be very valuable in the management of peanut allergy as they could help to identify peanut allergic patients who are at higher risk of developing life-threatening reactions and/or of reacting to low amounts of the allergens. The BAT reproduces very closely the phenotype of peanut-sensitised patients in relation to allergy versus tolerance, as reported in the previous chapter 4[165]. Basophils and mast cells are the effector cells of anaphylaxis. Basophils seem to be particularly relevant in food-induced anaphylaxis which often occurs without elevated serum tryptase. This observation suggests that the BAT could be used to safely assess the severity and the threshold of allergic reactions to peanut, as an *in vitro* surrogate for oral food challenges.

Different methods to express the results of the BAT, based on the allergen-induced dose-response curve, reflect different aspects of the basophil response, as described in Section 1.7.4. For example, the percentage of activated basophils measures basophil reactivity, whereas the concentration of allergen at which basophils become activated measures basophil sensitivity to the allergen[78]. I hypothesised that patients with severe reactions would show greater basophil reactivity and that patients who respond to lower doses of peanut allergen would show greater basophil sensitivity. I hypothesised that the higher percentage of basophils activated, the higher percentage of basophils degranulating and the higher the amount of vasoactive mediators released leading to more severe symptoms; and also that the threshold dose for basophil activation and degranulation *in vitro* during the BAT would correspond to the threshold dose *in vivo* during the challenges.

5.1 Severity and threshold study population

One hundred and twenty four patients were submitted to oral peanut challenges. Fifty two (42%) patients had a positive challenge to peanut and constituted the population of interest for the severity and threshold study. Three PA patients had non-responder basophils and were therefore excluded from further analyses. The study population (Table 5.1, n=49) was aged from 1.6 to 13 years (median age 5 years) and the majority (77%) had never ingested peanut prior to the challenge.

Table 5.1 Characteristics of the severity and threshold study population.

Values are expressed as number (percentage) or median (interquartile range).

Demographic features and investigations	Study population (n=49)
Age (years)	5.4 (4.7; 5.9)
Males - n (%)	34 (69.4%)
Symptom score	3 (3; 4)
Cumulative threshold dose of peanut protein (g)	0.1 (0.03; 0.63)
SPT to peanut (mm)	9 (7; 12)
Specific IgE to peanut (KU_A/l)	5.15 (0.51; 29.78)
Specific IgE to Ara h 1 (KU_A/l)	0.11 (0.02; 2.14)
Specific IgE to Ara h 2 (KU_A/l)	1.65 (0.20; 15.20)
Specific IgE to Ara h 3 (KU_A/l)	0.03 (0.01; 0.33)
Specific IgE to Ara h 8 (KU_A/l)	0.03 (0.01; 0.47)
Serum specific IgE to Ara h 9 (KU_A/l)	0.01 (0.01; 0.02)
Number of major peanut allergens bound by IgE	2 (1; 2)
Peanut-specific IgG4 (µg/l)	190.0 (120.0; 662.5)
Ratio of peanut-specific IgG4 to IgE	15.7 (5.7; 88.3)
Other food allergy - n (%)	47 (95.9%)
Atopic eczema - n (%)	40 (81.6%)
Asthma - n (%)	19 (38.8%)
Allergic rhinitis - n (%)	27 (55.1%)
Pollen allergy - n (%)	14 (28.6%)

5.2 Allergic reactions during the peanut challenges

5.2.1 Severity

Symptoms during the challenge ranged from mild oral symptoms to anaphylaxis. Twenty (41%) patients had severe reactions (Table 5.2). Nine (18.4%) patients required the administration of intramuscular adrenaline and 10 (77%) required the administration of intravenous fluid boluses. One patient (1.9%) had a biphasic reaction with symptoms appearing about 5 hours after the resolution of the allergic symptoms that had developed during the challenge.

Table 5.2 Severity of allergic reactions to peanut during the challenges

N=49

Symptom score	Number (%) of patients
1	4 (8.2%)
2	2 (4.1%)
3	23 (46.9%)
4	15 (30.6%)
5	5 (10.2%)

Severe reactors (i.e. symptom scores 4 or 5) had comparable SPT results (p=0.102) and higher levels of specific IgE to peanut (p=0.010), to Ara h 1 (p=0.021) and to Ara h 2 (p=0.003) compared to the patients who had mild/moderate reactions (i.e. symptom scores 1, 2 or 3). A greater number of peanut major allergens (Ara h 1, Ara h 2, Ara h 3) recognised by patients' IgE was also associated with severe reactions (p= 0.019).

Table 5.3 Characteristics of the study population according to the severity groups.

Values are expressed as number (percentage) or median (interquartile range). *p<0.001 not indicated as these characteristics formed the basis to classify the patients into severity and threshold groups. Significant p values are highlighted in bold. Abbreviation: SPT, skin prick test.

Demographic features and investigations	Severity groups		
	Mild/Moderate (n=29)	Severe (n=20)	p value
Age (years)	5.2 (4.4; 5.8)	5.4 (5.0; 6.2)	0.250
Males - n (%)	19 (65.5%)	15 (75.0%)	0.542
Symptom score	3 (3; 3)	4 (4; 5)	*
Cumulative threshold dose of peanut protein (g)	0.10 (0.33; 1.38)	0.10 (0.03; 0.38)	0.884
SPT to peanut (mm)	9 (4; 10)	10 (7; 13)	0.102
Specific IgE to peanut (KU_A/l)	1.86 (0.38; 12.85)	23.20 (5.18; 94.80)	**0.010**
Specific IgE to Ara h 1 (KU_A/l)	0.09 (0.01; 0.23)	0.20 (0.09; 11.58)	**0.021**
Specific IgE to Ara h 2 (KU_A/l)	0.68 (0.10; 2.67)	10.11 (1.48; 41.40)	**0.003**
Specific IgE to Ara h 3 (KU_A/l)	0.03 (0.01; 0.13)	0.05 (0.01; 0.98)	0.221
Specific IgE to Ara h 8 (KU_A/l)	0.03 (0.01; 0.23)	0.03 (0.01; 1.96)	0.582
Specific IgE to Ara h 9 (KU_A/l)	0.01 (0.01; 0.02)	0.01 (0.01; 0.07)	0.818
Number of major peanut allergens bound by IgE	1.0 (0; 2.0)	2.0 (1.0; 3.0)	**0.019**
Peanut-specific IgG4 (µg/l)	160 (120; 405)	380 (120; 1100)	0.235
Ratio of peanut-specific IgG4 to IgE	21.63 (5.78; 161.23)	11.26 (3.15; 76.75)	0.209
Other food allergy - n (%)	27 (93.1%)	20 (100.0%)	0.507
Atopic eczema - n (%)	23 (79.3%)	17 (85.0%)	0.720
Asthma - n (%)	11 (37.9%)	8 (40.0%)	1.0
Allergic rhinitis - n (%)	15 (51.7%)	12 (60.0 %)	0.771
Pollen allergy - n (%)	7 (24.1%)	7 (35.0%)	0.524

Patients who received intramuscular adrenaline had higher specific IgE to peanut (p=0.031) and to Ara h 2 (p=0.011) than patients who did not require adrenaline (Table 5.4).

Table 5.4 Demographic and clinical characteristics of study participants according to the requirement of intramuscular adrenaline.

Values are expressed as number (percentage) or median (interquartile range). Significant p values are highlighted in bold. Abbreviation: SPT, skin prick test.

Demographic and clinical features	Adrenaline required (n=9)	Adrenaline not required (n=40)	p value
Age (years)	5.15 (4.94; 6.31)	5.39 (4.63; 5.85)	0.786
Males - n (%)	6 (66.7%)	28 (70.0%)	1.0
Cumulative threshold dose of peanut protein (g)	0.10 (0.03; 0.24)	0.10 (0.03; 1.62)	0.579
SPT to peanut (mm)	10 (8; 16)	9 (5; 11)	0.111
Specific IgE to peanut (KU_A/l)	27.50 (15.93; 87.65)	2.74 (0.47; 16.53)	**0.031**
Specific IgE to Ara h 1 (KU_A/l)	0.13 (0.01; 3.25)	0.10 (0.02; 0.41)	0.667
Specific IgE to Ara h 2 (KU_A/l)	15.20 (9.21; 54.30)	1.09 (0.17; 6.60)	**0.011**
Specific IgE to Ara h 3 (KU_A/l)	0.05 (0.01; 1.10)	0.03 (0.01; 0.24)	0.333
Specific IgE to Ara h 8 (KU_A/l)	0.03 (0.02; 2.04)	0.02 (0.01; 0.28)	0.339
Specific IgE to Ara h 9 (KU_A/l)	0.02 (0.01; 0.04)	0.01 (0.01; 0.02)	0.653
Number of major peanut allergens bound by IgE	2 (1; 3)	2 (1; 2)	0.309
Peanut-specific IgG4 (µg/l)	590 (180; 1043)	160 (120; 575)	0.135
Ratio of peanut-specific IgG4 to IgE	10.05 (1.90; 149.91)	17.62 (5.88; 88.33)	0.346
Other food allergy - n (%)	9 (100.0%)	38 (95.0%)	1.0
Atopic eczema - n (%)	7 (77.8%)	33 (82.5%)	0.663
Asthma - n (%)	3 (33.3%)	16 (40.0%)	1.0
Allergic rhinitis - n (%)	7 (77.8%)	20 (50.0%)	0.159
Pollen allergy - n (%)	4 (44.4%)	10 (25.0%)	0.254

5.2.2 Threshold

The cumulative threshold dose of peanut protein varied between 0.033 and 9.35 g (median=0.1 g). Twenty-eight (57%) patients reacted to 0.1 g or less of peanut protein during the oral food challenge (Table 5.5).

Table 5.5 Threshold of allergic reactions to peanut during the challenges

N=49

Cumulative threshold dose of peanut protein (g)	Number (%) of patients
0.033	19 (39%)
>0.033 <0.1	2 (4%)
0.1	7 (14%)
>0.1 <4	14 (29%)
≥4	7 (14%)

Patients with lower threshold had larger wheals on SPT to peanut (p=0.021) and higher levels of specific IgE to peanut (p=0.026) and to Ara h 2 (p=0.032) than patients who reacted to more than 0.1 g of peanut protein (Table 5.6). Interestingly, patients with a higher cumulative peanut threshold dose had a higher ratio of peanut-specific IgG4 to IgE (p=0.011).

Table 5.6 Characteristics of the study population according to the threshold groups.

Values are expressed as number (percentage) or median (interquartile range). *p<0.001 not indicated as these characteristics formed the basis to classify the patients into the threshold groups. Significant p values are highlighted in bold. Abbreviation: SPT, skin prick test.

Demographic features and investigations	Threshold groups according to the cumulative threshold dose		
	≤0.1g peanut protein (n=28)	>0.1g peanut protein (n=21)	p value
Age (years)	5.3 (4.8; 5.8)	5.4 (4.6; 6.1)	0.928
Males - n (%)	18 (64.3%)	16 (76.2%)	0.533
Cumulative threshold dose of peanut protein (g)	0.03 (0.03; 0.09)	0.92 (0.37; 4.38)	*
SPT to peanut (mm)	10 (8; 13)	8 (4; 11)	**0.021**
Specific IgE to peanut (KU_A/l)	7.17 (1.73; 75.80)	1.33 (0.38; 12.35)	**0.026**
Specific IgE to Ara h 1 (KU_A/l)	0.18 (0.04; 8.67)	0.09 (0.01; 0.15)	0.051
Specific IgE to Ara h 2 (KU_A/l)	5.05 (1.06; 46.80)	0.46 (0.12; 7.36)	**0.032**
Specific IgE to Ara h 3 (KU_A/l)	0.04 (0.01; 0.89)	0.02 (0.01; 0.05)	0.069
Specific IgE to Ara h 8 (KU_A/l)	0.03 (0.01; 0.28)	0.02 (0.01; 2.04)	1.0
Serum IgE to Ara h 9 (KU_A/l)	0.01 (0.01; 0.04)	0.01 (0.01; 0.02)	0.856
Number of major peanut allergens bound by IgE	2 (1; 3)	1 (1; 2)	0.089
Peanut-specific IgG4 (µg/l)	140 (120; 800)	290 (130; 660)	0.425
Ratio of peanut-specific IgG4 to IgE	8.6 (2.3; 66.1)	48.9 (15.5; 159.4)	**0.011**
Other food allergy - n (%)	28 (100.0%)	19 (90.5%)	0.179
Atopic eczema - n (%)	25 (89.3%)	15 (71.4%)	0.146
Asthma - n (%)	11 (39.3%)	8 (38.1%)	1.0
Allergic rhinitis - n (%)	19 (67.9%)	8 (38.1%)	**0.048**
Pollen allergy - n (%)	8 (28.6%)	6 (28.6%)	1.0

Classifying patients according to the *discrete* threshold dose of peanut protein at the time of reaction yielded similar findings (Table 5.7).

Table 5.7 Characteristics of the study population grouped according to the discrete threshold doses of peanut protein on oral food challenges.

Values are expressed as number (percentage) or median (interquartile range). *p<0.001 not indicated as these characteristics formed the basis to classify the patients into the threshold groups. Significant p values are highlighted in bold. Abbreviation: SPT, skin prick test.

Demographic features and investigations	Threshold groups according to the discrete threshold dose		
	≤0.1g peanut protein (n=31)	>0.1g peanut protein (n=18)	p value
Age (years)	5.4 (4.9; 5.9)	5.3 (4.5; 5.8)	0.799
Males - n (%)	20 (65%)	14 (78%)	0.521
Discrete threshold dose of peanut protein (g)	0.03 (0.03; 0.10)	1.0 (0.46; 3.13)	*
SPT to peanut (mm)	10 (8; 13)	4 (4; 10)	**0.005**
Specific IgE to peanut (KU_A/l)	10.34 (1.85; 67.40)	0.80 (0.36; 6.63)	**0.009**
Specific IgE to Ara h 1 (KU_A/l)	0.20 (0.04; 4.61)	0.09 (0.01; 0.13)	**0.031**
Specific IgE to Ara h 2 (KU_A/l)	5.23 (1.10; 39.53)	0.38 (0.08; 1.26)	**0.005**
Specific IgE to Ara h 3 (KU_A/l)	0.04 (0.02; 0.91)	0.01 (0.01; 0.04)	**0.015**
Specific IgE to Ara h 8 (KU_A/l)	0.04 (0.01; 1.96)	0.02 (0.01; 0.04)	0.178
Serum IgE to Ara h 9 (KU_A/l)	0.01 (0.01; 0.08)	0.01 (0.01; 0.02)	0.612
Number of major peanut allergens bound by IgE	2.0 (1.0; 3.0)	1.0 (0.5; 2.0)	**0.008**
Peanut-specific IgG4 (µg/l)	190 (120; 818)	240 (100; 648)	0.830
Ratio of peanut-specific IgG4 to IgE	8.7 (2.9; 51.5)	83.8 (16.3; 189.5)	**0.005**
Other food allergy - n (%)	31 (100%)	16 (34%)	0.130
Atopic eczema - n (%)	28 (70%)	12 (30%)	0.058
Asthma - n (%)	12 (63%)	7 (37%)	1.0
Allergic rhinitis - n (%)	21 (78%)	6 (22%)	**0.036**
Pollen allergy - n (%)	9 (64%)	5 (28%)	1.0

5.3 Basophil activation test markers of severity and threshold

BAT parameters were assessed in relation to the severity and the threshold dose of reaction to peanut during the oral food challenges. Table 5.8 shows the results for the studied BAT parameters in the study population overall.

Table 5.8 Results for the various BAT parameters in the total study population

N=49. Values are expressed as median (interquartile range). CD_{sens} was calculated based on the CD63 dose-response curve. Numbers in the left column indicate the concentration of peanut extract in ng/ml. Abbreviation: AUC CD63, area under the dose-response curve considering the percentage of CD63-positive basophils; BAT, basophil activation test; EC50, half-maximal effective concentration.

BAT to peanut	Study population
%CD63+ Peanut 0.1	0.63 (0; 3.05)
%CD63+ Peanut 1	2.38 (0.90; 15.44)
%CD63+ Peanut 10	18.16 (4.45; 51.90)
%CD63+ Peanut 100	21.78 (8.44; 51.88)
%CD63+ Peanut 1,000	23.53 (7.70; 43.37)
%CD63+ Peanut 10,000	34.36 (12.32; 55.44)
Mean %CD63 Peanut 10-100	16.94 (6.29; 52.38)
AUC CD63 Peanut	86.96 (46.82; 207.38)
Maximal %CD63+ to peanut	39.90 (15.20; 67.39)
%CD63+ peanut/anti-IgE	0.82 (0.32; 1.32)
EC_{50} (ng/ml) - CD63	10 (1; 10)
CD_{sens}	12.97 (1.96; 85.91)

5.3.1 Severity of allergic reactions to peanut is associated with greater allergen-specific basophil reactivity

Patients with severe reactions to peanut during the oral food challenge had a higher proportion of CD63-positive basophils at concentrations of peanut extract ranging from 0.1 to 10,000 ng/ml compared to peanut allergic patients with mild/moderate reactions (p=0.003-0.049; Figure 5.1 and Table 5.9).

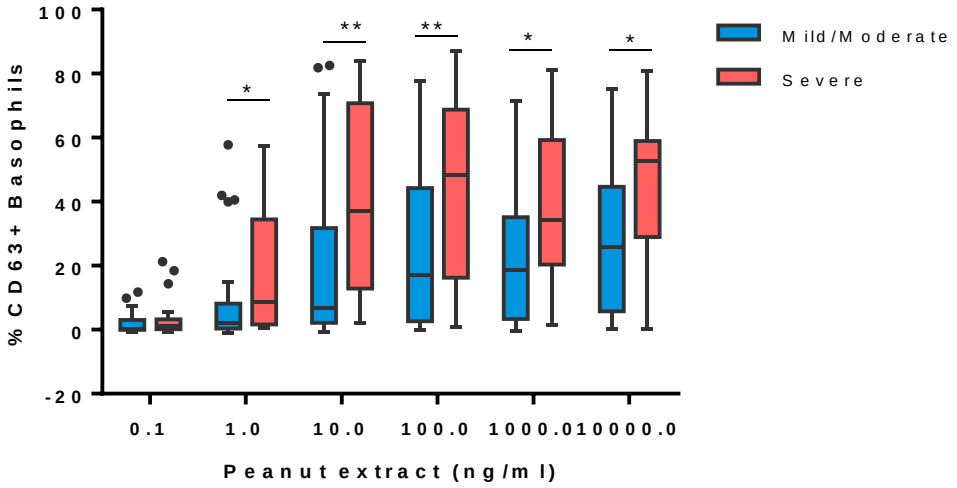

Figure 5.1 Peanut-dose-response of basophil activation in patients with severe versus mild/moderate reactions.

**p<0.01 *p<0.05.

Table 5.9 BAT parameters associated with the severity of allergic reactions to peanut.

Values are expressed as median (interquartile range). CD_{sens} was calculated based on the CD63 dose-response curve. Numbers in the left column indicate the concentration of peanut extract in ng/ml. Significant p values are highlighted in bold. Abbreviation: AUC CD63, area under the dose-response curve considering the percentage of CD63-positive basophils.

BAT to peanut	Severity groups		
	Mild/Moderate (n=29)	Severe (n=20)	p value
%CD63+ Peanut 0.1	0.36 (0; 3.05)	1.03 (0.16; 3.22)	0.215
%CD63+ Peanut 1	2.13 (0.36; 8.12)	8.60 (1.68; 34.41)	**0.016**
%CD63+ Peanut 10	6.65 (2.15; 31.73)	37.04 (12.80; 70.71)	**0.009**
%CD63+ Peanut 100	17.11 (2.60; 44.28)	48.44 (16.24; 68.79)	**0.003**
%CD63+ Peanut 1,000	18.56 (3.37; 35.18)	34.33 (20.31; 59.27)	**0.049**
%CD63+ Peanut 10,000	25.86 (5.74; 44.68)	52.79 (28.92; 58.93)	**0.012**
Mean %CD63 Peanut 10-100	13.26 (2.56; 38.05)	41.31 (10.23; 67.36)	**0.012**
AUC CD63 Peanut	66.44 (27.64; 164.91)	159.14 (78.84; 240.42)	**0.016**
Maximal %CD63+ to peanut	27.74 (9.99; 56.23)	59.49 (31.47; 75.85)	**0.025**
%CD63+ peanut/anti-IgE	0.53 (0.15; 0.85)	1.32 (0.92; 1.55)	**<0.001**
EC_{50} (ng/ml) - CD63	10 (1; 100)	10 (1; 10)	0.058
CD_{sens}	5.37 (0.99; 50.66)	32.59 (11.62; 87.97)	**0.023**

The best basophil markers for the severity of allergic reactions were the ratio of the percentage of CD63-positive basophils at 100 ng/ml of peanut to the percentage of CD63-positive basophils following stimulation with anti-IgE (CD63 peanut/anti-IgE, p<0.001; Figure 5.2) and the percentage of CD63-positive basophils at 100 ng/ml of peanut extract (p=0.008). The latter marker was previously identified as optimal for the diagnosis of peanut allergy[165] and was the best discriminator of the patients who had severe reactions requiring the administration of intramuscular adrenaline (Table 5.10).

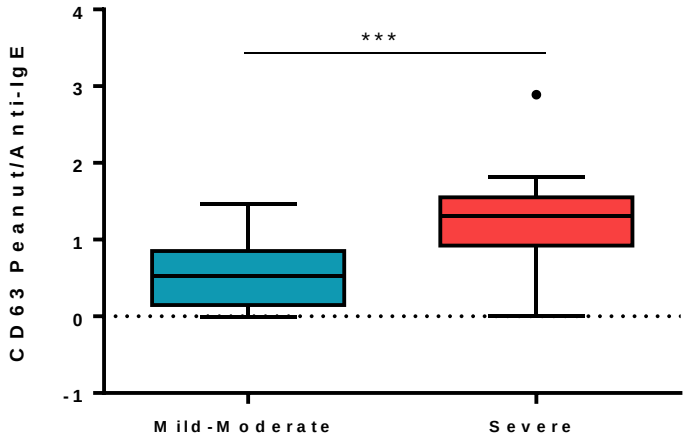

Figure 5.2 Best BAT parameter associated with the severity of allergic reactions.

The best BAT parameter to distinguish between patients with severe versus non-severe reactions to peanut was the ratio of the %CD63+ basophils at 100 ng/ml of peanut to the %CD63+ basophils following stimulation with anti-IgE. *** p<0.001.

Table 5.10 BAT parameters according to the requirement of intramuscular adrenaline.

Values are expressed as median (interquartile range). CD_{sens} was calculated based on the CD63 dose-response curve. Numbers in the left column indicate the concentration of peanut extract in ng/ml. Significant p values are highlighted in bold. Abbreviation: AUC CD63, area under the dose-response curve considering the percentage of CD63-positive basophils.

BAT parameters	Adrenaline required (n=9)	Adrenaline not required (n=40)	p value
%CD63+ Peanut 0.1	3.31 (1.98; 12.96)	0.33 (0; 1.18)	**0.001**
%CD63+ Peanut 1	5.65 (8.60; 45.65)	1.95 (0.67; 9.42)	**0.001**
%CD63+ Peanut 10	59.63 (37.04; 73.93)	10.69 (2.46; 35.16)	**0.002**
%CD63+ Peanut 100	68.01 (45.09; 74.66)	17.59 (6.08; 46.41)	**<0.001**
%CD63+ Peanut 1,000	49.65 (35.13; 64.49)	20.31 (3.54; 34.33)	**0.002**
%CD63+ Peanut 10,000	57.30 (39.34; 64.54)	27.43 (6.66; 49.64)	**0.007**
Mean %CD63 Peanut 10-100	62.27 (35.72; 72.63)	13.67 (5.32; 41.26)	**0.003**
AUC CD63 Peanut	223.95 (131.91; 276.69)	76.74 (31.88; 183.94)	**0.001**
Maximal %CD63+ to peanut	70.31 (62.68; 80.26)	31.91 (11.29; 58.14)	**0.001**
%CD63+ peanut/anti-IgE	1.34 (0.99; 1.45)	0.64 (0.20; 1.21)	**0.010**
EC_{50} (ng/ml) - CD63	1.0 (1.0; 10.0)	10.0 (1.0; 100.0)	0.066
CD_{sens}	51.27 (22.78; 110.65)	9.32 (1.49; 53.74)	**0.014**

5.3.2 Basophil sensitivity indicates the threshold of allergic reactions to peanut

The dose-response for peanut-induced basophil activation of patients with lower cumulative peanut threshold on oral food challenge was shifted to the left compared to the dose-response of patients with a higher cumulative peanut threshold (Figure 5.3).

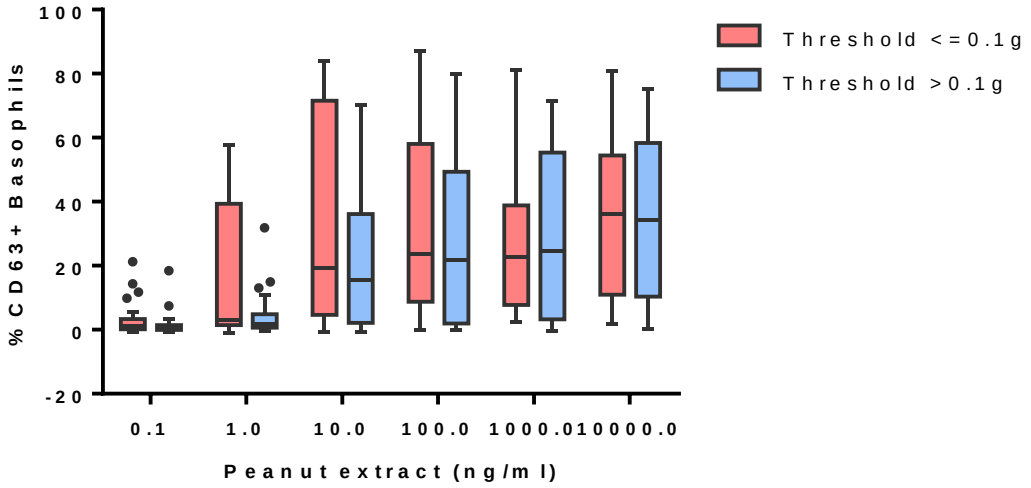

Figure 5.3 Peanut-dose-response of basophil activation in patients with low versus high threshold of reactivity to peanut.

Peanut allergic patients with lower threshold on oral food challenge had higher basophil sensitivity as expressed by a higher CD_{sens} (p=0.005) and a correspondingly lower EC_{50} (p=0.019; Table 5.11 and Figure 5.4). Similar differences were found when considering the discrete threshold doses.

Table 5.11 BAT parameters associated with the threshold of allergic reactions to peanut.

Values are expressed as median (interquartile range). CD_{sens} was calculated based on the CD63 dose-response curve. Numbers in the left column indicate the concentration of peanut extract in ng/ml. Significant p values are highlighted in bold. Abbreviation: AUC CD63, area under the dose-response curve considering the percentage of CD63-positive basophils.

BAT to peanut	Threshold groups		
	≤0.1g peanut protein (n=28)	>0.1g peanut protein (n=21)	p value
%CD63+ Peanut 0.1	1.07 (0.14; 3.34)	0.36 (0; 1.43)	0.140
%CD63+ Peanut 1	3.08 (1.41; 39.38)	1.76 (0.68; 4.85)	0.090
%CD63+ Peanut 10	19.33 (4.67; 71.52)	15.54 (2.15; 36.12)	0.163
%CD63+ Peanut 100	23.60 (8.73; 58.03)	21.78 (1.99; 49.36)	0.505
%CD63+ Peanut 1,000	22.86 (7.70; 38.88)	24.57 (3.30; 55.36)	0.818
%CD63+ Peanut 10,000	36.08 (10.96; 54.47)	34.36 (10.40; 58.37)	0.888
Mean %CD63 Peanut 10-100	10.18 (5.16; 16.09)	20.41 (4.36; 43.42)	0.419
AUC CD63 Peanut	92.96 (34.24; 231.34)	86.96 (52.84; 196.60)	0.671
Maximal %CD63+ to peanut	47.59 (15.12; 74.75)	34.66 (13.08; 62.81)	0.303
%CD63+ peanut/anti-IgE	0.79 (0.44; 1.26)	0.83 (0.09; 1.35)	0.716
EC_{50} (ng/ml) - CD63	1.0 (1.0; 32.5)	10.0 (10.0; 10.0)	**0.019**
CD_{sens}	32.59 (2.58; 113.75)	5.37 (0.80; 13.13)	**0.005**

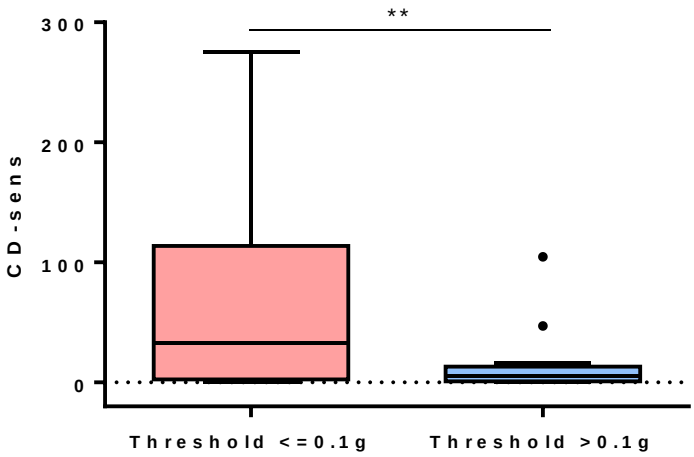

Figure 5.4 Best BAT parameter associated with the threshold of allergic reactions

The best BAT parameter to distinguish between patients with low versus high threshold of reactivity to peanut was CD_{sens} determined using the CD63 dose-response curve. **p<0.01.

5.3.3 Logistic regression analyses to assess different parameters of severity and threshold

As various parameters other than BAT varied with the severity and the threshold of allergic reactions to peanut (Table 5.9 and Table 5.11), logistic regression analyses were conducted to assess which parameters were independently associated with severity and threshold (Table 5.12). Following multivariable analyses, only the basophil activation markers were retained for severity and for threshold meaning that the BAT alone was more discriminative in predicting the severity (CD63 peanut/anti-IgE, p=0.001) and the threshold (CD_{sens}, p=0.020) of allergic reactions to peanut than the other parameters or the combination of BAT with the other parameters.

Table 5.12 Univariable and multivariable analyses of factors associated with the severity and threshold of allergic reactions to peanut.

*All variables were re-tested by forward multivariable logistic regression and only variables contributing to the model ($p<0.05$) were retained. CD_{sens} refers to CD_{sens} calculated using the CD63 dose-response curve.

Variable	**Severe allergic reaction**		**Low threshold of reactivity**	
	OR (95% CI)	**p value**	**OR (95% CI)**	**p value**
Univariable analysis				
%CD63+ peanut/anti-IgE	0.111 (0.026; 0.478)	**0.001**	-	-
CD_{sens}	-	-	1.027 (1.004; 1.050)	**0.020**
SPT	-	-	1.231 (1.033; 1.466)	**0.020**
Specific IgE to peanut	1.014 (1.0; 1.029)	0.056	1.022 (1.0; 1.045)	0.053
Ara h 1-specific IgE	1.031 (0.985; 1.079)	0.186	1.016 (0.975; 1.058)	0.459
Ara h 2-specific IgE	1.034 (0.999; 1.070)	0.054	1.036 (0.994; 1.080)	0.090
Ara h 3-specific IgE	-	-	3.391 (0.590; 19.501)	0.171
Number of major peanut allergens bound by IgE	2.342 (1.144; 4.791)	**0.020**	1.712 (0.896; 3.272)	0.104
Ratio peanut-sIgG4/IgE	-	-	0.999 (0.997; 1.001)	0.283
Allergic rhinitis	-	-	3.431 (1.049; 11.222)	**0.041**
Multivariable analysis*				
%CD63+ peanut/anti-IgE	**0.111 (0.026; 0.478)**	**0.001**	-	-
CD_{sens}	-	-	**1.027 (1.004; 1.050)**	**0.020**

5.4 Basophil activation test cut-offs for severity and threshold

The ratio of the percentage of CD63-positive basophils following stimulation with 100 ng/ml of peanut extract to the percentage of CD63-positive basophils following stimulation with anti-IgE was chosen as the BAT parameter to reflect the severity of allergic reactions. A ratio greater or equal to 1.3 increased the proportion of severe reactors by 3-fold (relative risk (RR) =3.4; 95% CI=1.8-6.2) compared to patients with less than 1.3 for the ratio of %CD63+ peanut/anti-IgE (p=0.001, Figure 5.5).

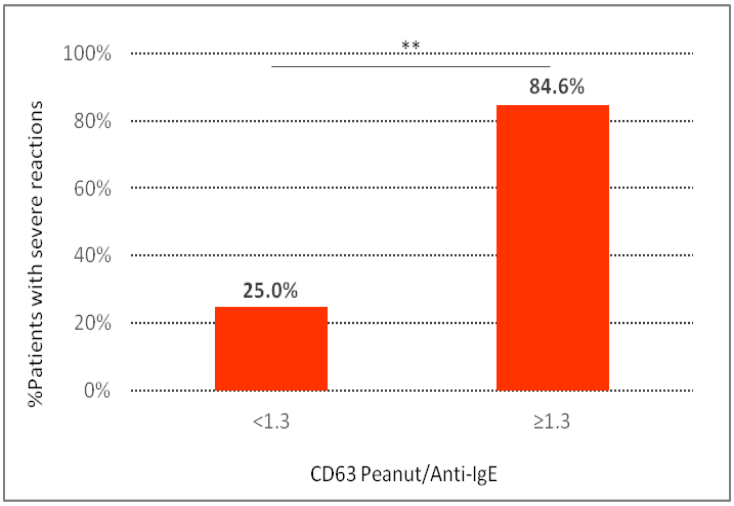

Figure 5.5 Proportion of patients with severe reactions according to the 75th percentile of basophil reactivity

Basophil reactivity was measured by %CD63+ peanut/anti-IgE. **p<0.01.

Patients with CD_{sens} greater or equal to 84.0 were two times more likely to react to low amounts of peanut (RR=1.9; 95%CI=1.3-2.8) compared to patients with lower values of CD_{sens} and thus with a higher threshold of reactivity to peanut (p=0.014, Figure 5.6.).

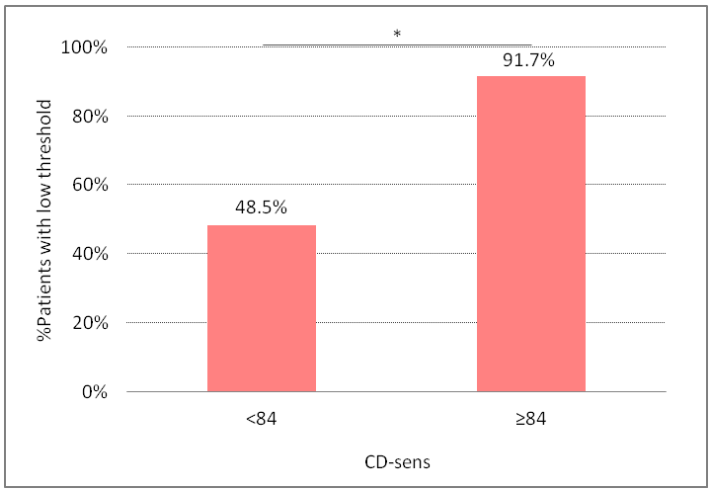

Figure 5.6 Proportion of patients with lower threshold of reactivity to peanut according to the 75th percentile of basophil sensitivity

Basophil sensitivity was measured by CD_{sens}. *p=0.05.

BAT cut-offs to estimate the severity (Figure 5.7 and Table 5.13) and the threshold (Figure 5.8 and Table 5.14) of peanut allergic reactions were also determined by ROC curve analyses.

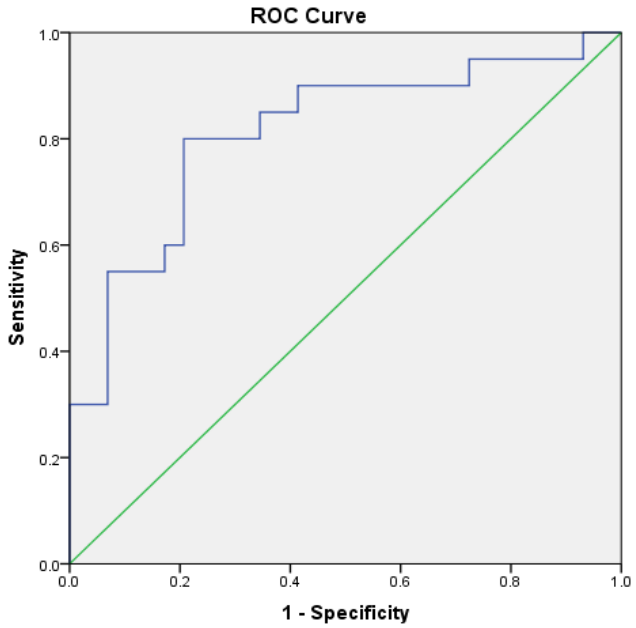

Figure 5.7 Receiver operating characteristic (ROC) curve for the use of %CD63+ peanut/anti-IgE for estimation of the severity of allergic reactions to peanut during challenges.

AUC (95% CI)=0.81 (0.68; 0.91)

Table 5.13 Negative, optimal and positive cut-offs for the use of the ratio between the proportion of CD63-positive basophils following stimulation with peanut and with anti-IgE for estimation of the severity of allergic reactions to peanut during challenges.

Cut-offs for %CD63+ peanut/anti-IgE	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)
> 0.60	90.0 (68.3; 98.8)	58.6 (38.9; 76.5)	60.0 (40.6; 77.3)	89.5 (66.96; 98.7)
> 0.87	80.0 (56.3; 94.3)	79.3 (60.3; 92.0)	72.7 (49.8; 89.3)	85.2 (66.3; 95.8)
> 1.42	30.0 (11.9; 54.3)	96.6 (82.2; 99.9)	85.7 (42.1; 99.6)	66.7 (50.5; 80.4)

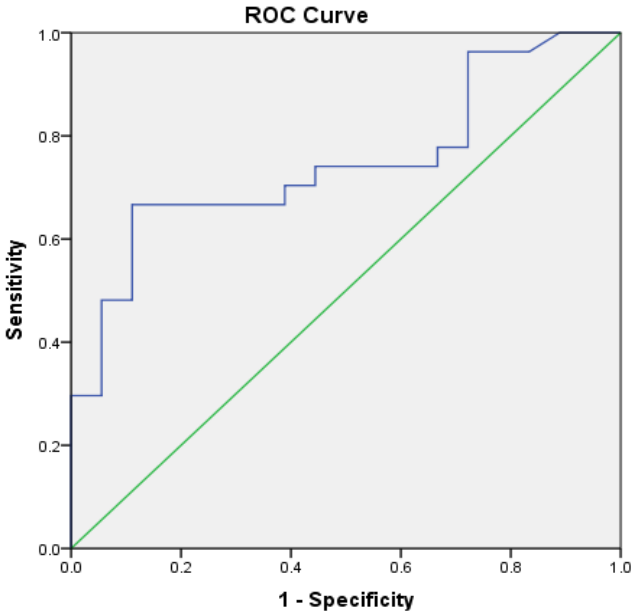

Figure 5.8 Receiver operating characteristic (ROC) curve for the use of CD_{sens} for the estimation of the threshold of allergic reactions to peanut during challenges.

[AUC (95% CI)=0.75 (0.60; 0.87)]

Table 5.14 Negative, optimal and positive cut-offs for the use of CD_{sens} for the estimation of the threshold of allergic reactions to peanut during challenges.

Cut-offs for CD_{sens}	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)
> 0.9	96.3 (81.0; 99.9)	27.8 (9.1; 53.5)	66.7 (49.8; 80.9)	83.3 (35.9; 99.6)
> 16.5	66.7 (46.0; 83.5)	88.9 (65.3; 98.6)	90.0 (68.3; 98.8)	64.0 (42.5; 82.0)
> 104.7	29.6 (13.8; 52.2)	100.0 (81.5; 100.0)	100.0 (63.1; 100.0)	48.6 (31.9; 65.6)

5.5 Correlation between severity and threshold

Clinically, symptom score and threshold dose were not correlated (Rs= -0.067, p=0.645, Table 5.15). However, the basophil markers of severity (CD63 peanut/anti-IgE) and of threshold (CD_{sens}) were strongly correlated (Rs=0.60; p<0.001,).

Table 5.15 Correlations between severity (as measured by symptom score) and threshold (as measured by cumulative threshold dose) of allergic reactions to peanut and different diagnostic tests.

N=44. Spearman correlation coefficient and p values are indicated. Significant correlations are highlighted in bold. CD_{sens} was calculated based on the CD63 dose-response curve. Abbreviation: SPT, skin prick test.

	Symptom score	Cumulative threshold dose
Symptom score	-	r_S= -0.067 p=0.645
Time to reaction (minutes)	r_S=0.040 p=0.790	**r_S=0.294** **p=0.043**
SPT (mm)	**r_S=0.417** **p=0.003**	**r_S= -0.394** **p=0.005**
Peanut IgE specific activity (%)	**r_S=0.398** **p=0.008**	r_S= -0.235 p=0.124
Peanut-specific IgE (KU_A/l)	**r_S=0.494** **p<0.001**	**r_S= -0.375** **p=0.009**
Ara h 1-specific IgE (KU_A/l)	**r_S=0.406** **p=0.007**	r_S= -0.310 p=0.043
Ara h 2-specific IgE (KU_A/l)	**r_S=0.536** **p<0.001**	**r_S= -0.420** **p=0.005**
Ara h 3-specific IgE (KU_A/l)	r_S=0.225 p=0.152	r_S= -0.302 p=0.052
Ara h 8-specific IgE (KU_A/l)	r_S=0.217 p=0.167	r_S= -0.051 p=0.749
Ara h 9-specific IgE (KU_A/l)	r_S=0.131 p=0.415	r_S=0.095 p=0.556
Peanut-specific IgG4 (µg/ml)	r_S=0.247 p=0.106	r_S=0.139 p=0.367
Ratio peanut specific IgG4/IgE	r_S= -0.276 p=0.070	**r_S=0.453** **p=0.002**
%CD63+ peanut/anti-IgE	**r_S=0.548** **p<0.001**	r_S= -0.200 p=0.168
CD_{sens}	**r_S=0.391** **p=0.008**	**r_S= -0.518** **p<0.001**

5.6 Classification of the severity of allergic reactions to peanut using different severity scores

The severity of allergic reactions to peanut was classified according to severity scores other than the one described by Ewan et al[148] (Table 5.3 and Table 5.9), namely the one described by Mueller et al[172] (Table 5.16), the one described by Brown et al[173] (Table 5.17), the one described by Sampson et al[174] (Table 5.18) and the one described by Van der Zee et al[175] (Table 5.19). The comparison of severity groups defined according to the different criteria showed similar although not perfectly overlapping results, with SPT, specific IgE to peanut and to Ara h 2 and number of allergens as well as markers of basophil reactivity being associated with severity considering the majority of severity scores.

Table 5.16 Demographic, clinical and BAT parameters between severe and non-severe reactors classified according to the severity score adopted by Mueller et al[172].

Values are expressed as number (percentage) or median (interquartile range). Significant p values are highlighted in bold. CD_{sens} was calculated based on the CD63 dose-response curve. Numbers in the left column indicate the concentration of peanut extract in ng/ml. Abbreviations: SPT, skin prick test; AUC CD63, area under the dose-response curve considering the percentage of CD63-positive basophils.

Demographic and clinical features	Mild/Moderate (n=26)	Severe (n=23)	p value
Age (years)	5.2 (4.0; 5.8)	5.4 (5.0; 6.2)	0.176
Males - n (%)	17 (65%)	17 (74%)	0.552
Cumulative threshold dose of peanut protein (g)	0.08 (0.03; 1.15)	0.10 (0.03; 0.50)	0.594
SPT to peanut (mm)	9 (4; 10)	10 (7; 13)	**0.045**
Specific IgE to peanut (KU_A/l)	1.81 (0.36; 12.03)	15.35 (4.17; 61.65)	**0.017**
Specific IgE to Ara h 1 (KU_A/l)	0.09 (0.01; 0.28)	0.17 (0.06; 7.99)	0.051
Specific IgE to Ara h 2 (KU_A/l)	0.73 (0.09; 2.95)	7.31 (0.57; 33.48)	**0.011**
Specific IgE to Ara h 3 (KU_A/l)	0.03 (0.01; 0.18)	0.04 (0.01; 0.89)	0.340
Specific IgE to Ara h 8 (KU_A/l)	0.02 (0.01; 0.14)	0.03 (0.01; 2.01)	0.324
Specific IgE to Ara h 9 (KU_A/l)	0.01 (0.01; 0.02)	0.01 (0.01; 0.04)	0.395
Number of major peanut allergens bound by IgE	1.0 (0; 2.0)	2.0 (1.0; 3.0)	**0.024**
Peanut-specific IgG4 (µg/l)	150 (115; 363)	335 (128; 928)	0.151
Ratio of peanut-specific IgG4 to IgE	19.93 (5.64; 147.66)	12.64 (5.09; 77.76)	0.342
Other food allergy - n (%)	24 (92%)	23 (100%)	0.491
Atopic eczema - n (%)	21 (81%)	19 (83%)	1.0
Asthma - n (%)	11 (42%)	8 (35%)	0.770
Allergic rhinitis - n (%)	14 (54%)	13 (57%)	1.0
Pollen allergy - n (%)	6 (23%)	8 (35%)	0.528
BAT parameters	Mild/Moderate (n=26)	Severe (n=23)	p value
%CD63+ Peanut 0.1	0.3 (0; 3.0)	1.0 (0.1; 3.3)	0.225
%CD63+ Peanut 1	2.2 (0.4; 10.8)	3.8 (1.5; 31.8)	0.062

%CD63+ Peanut 10	7.7 (2.2; 37.8)	27.5 (9.1; 70.3)	0.050
%CD63+ Peanut 100	17.6 (2.8; 45.1)	34.7 (9.7; 68.0)	**0.021**
%CD63+ Peanut 1,000	19.9 (3.5; 36.7)	29.9 (10.1; 52.1)	0.131
%CD63+ Peanut 10,000	23.7 (6.0; 44.3)	45.4 (27.1; 58.7)	**0.025**
Mean %CD63 Peanut 10-100	13.4 (4.7; 43.0)	26.6 (8.1; 62.3)	0.075
AUC CD63 Peanut	69.4 (22.1; 192.4)	115.8 (66.4; 223.9)	**0.032**
Maximal %CD63+ to peanut	26.8 (10.6; 61.5)	54.5 (30.5; 75.1)	0.060
%CD63+ peanut/anti-IgE	0.53 (0.15; 0.82)	1.18 (0.92; 1.53)	**<0.001**
EC_{50} (ng/ml) - CD63	10 (1; 100)	10 (1; 10)	0.103
CD_{sens}	4.83 (1.01; 108.58)	28.62 (11.51; 84.87)	0.056

Table 5.17 Demographic, clinical and BAT parameters between severe and non-severe reactors classified according to the severity score adopted by Brown et al[173].

Values are expressed as number (percentage) or median (interquartile range). Significant p values are highlighted in bold. CD_{sens} was calculated based on the CD63 dose-response curve. Numbers in the left column indicate the concentration of peanut extract in ng/ml. Abbreviations: SPT, skin prick test; AUC CD63, area under the dose-response curve considering the percentage of CD63-positive basophils.

Demographic and clinical features	Mild/Moderate (n=8)	Severe (n=41)	p value
Age (years)	4.0 (3.1; 5.3)	5.4 (4.9; 5.9)	**0.030**
Males - n (%)	6 (75%)	28 (68%)	1.0
Cumulative threshold dose of peanut protein (g)	0.24 (0.04; 3.79)	0.10 (0.03; 0.38)	0.472
SPT to peanut (mm)	5 (4; 9)	9 (7; 13)	**0.030**
Specific IgE to peanut (KU_A/l)	0.41 (0.21; 2.75;	6.23 (1.09; 31.32)	**0.027**
Specific IgE to Ara h 1 (KU_A/l)	0.02 (0.01; 0.17)	0.13 (0.03; 3.16)	0.074
Specific IgE to Ara h 2 (KU_A/l)	0.03 (0.01; 0.20)	3.53 (0.43; 19.19)	**0.002**
Specific IgE to Ara h 3 (KU_A/l)	0.03 (0.01; 0.16)	0.03 (0.01; 0.47)	0.716
Specific IgE to Ara h 8 (KU_A/l)	0.01 (0.01; 0.04)	0.03 (0.01; 0.47)	0.170
Specific IgE to Ara h 9 (KU_A/l)	0.01 (0.01; 0.02)	0.01 (0.01; 0.03)	0.573
Number of major peanut allergens bound by IgE	1.0 (0; 2.0)	2.0 (1.0; 3.0)	**0.035**
Peanut-specific IgG4 (µg/l)	140 (83; 468)	215 (128; 703)	0.337
Ratio of peanut-specific IgG4 to IgE	98.17 (5.20; 705.56)	14.20 (5.74; 77.76)	0.259
Other food allergy - n (%)	6 (75%)	41 (100%)	**0.024**
Atopic eczema - n (%)	6 (75%)	34 (83%)	0.628
Asthma - n (%)	2 (25%)	17 (42%)	0.458
Allergic rhinitis - n (%)	2 (25%)	25 (61%)	0.117
Pollen allergy - n (%)	0 (0%)	14 (34%)	0.085
BAT parameters	Mild/Moderate (n=8)	Severe (n=41)	p value
%CD63+ Peanut 0.1	0 (0; 0.3)	1.0 (0.2; 3.2)	**0.011**
%CD63+ Peanut 1	1.1 (0; 2.2)	3.1 (1.3; 16.5)	0.070

%CD63+ Peanut 10	3.0 (0; 16.2)	22.7 (4.9; 59.5)	**0.025**
%CD63+ Peanut 100	10.2 (0.1; 38.6)	26.4 (8.9; 56.2)	**0.045**
%CD63+ Peanut 1,000	10.5 (0.3; 30.7)	25.7 (9.0; 48.4)	**0.043**
%CD63+ Peanut 10,000	18.7 (0.8; 36.7)	40.7 (15.7; 57.5)	**0.033**
Mean %CD63 Peanut 10-100	9.1 (0; 28.5)	21.2 (7.0; 59.1)	0.055
AUC CD63 Peanut	69.4 (4.3; 129.4)	89.1 (48.5; 211.1)	0.123
Maximal %CD63+ to peanut	25.6 (1.2; 49.2)	42.9 (17.1; 69.7)	0.088
%CD63+ peanut/anti-IgE	0.20 (0; 1.08)	0.87 (0.47; 1.34)	**0.035**
EC_{50} (ng/ml) - CD63	10 (3; 78)	10 (1; 10)	0.699
CD_{sens}	1.52 (0.46; 10.27)	18.57 (2.60; 91.68)	0.056

Table 5.18 Demographic, clinical and BAT parameters between severe and non-severe reactors classified according to the severity score adopted by Sampson et al[174].

Values are expressed as number (percentage) or median (interquartile range). Significant p values are highlighted in bold. CD_{sens} was calculated based on the CD63 dose-response curve. Numbers in the left column indicate the concentration of peanut extract in ng/ml. Abbreviations: SPT, skin prick test; AUC CD63, area under the dose-response curve considering the percentage of CD63-positive basophils.

Demographic and clinical features	Mild/Moderate (n=28)	Severe (n=21)	p value
Age (years)	5.3 (4.6; 5.9)	5.4 (4.9; 5.8)	0.578
Males - n (%)	18 (64%)	16 (76%)	0.533
Cumulative threshold dose of peanut protein (g)	0.08 (0.03; 0.78)	0.1 (0.3; 0.63)	0.627
SPT to peanut (mm)	9 (6; 10)	10 (7; 13)	0.306
Specific IgE to peanut (KU_A/l)	1.97 (0.38; 13.68)	18.35 (2.08; 83.75)	**0.029**
Specific IgE to Ara h 1 (KU_A/l)	0.09 (0.01; 0.26)	0.18 (0.02; 8.67)	0.099
Specific IgE to Ara h 2 (KU_A/l)	0.90 (0.19; 2.81)	9.21 (0.29; 37.10)	**0.040**
Specific IgE to Ara h 3 (KU_A/l)	0.03 (0.01; 0.16)	0.04 (0.01; 0.94)	0.386
Specific IgE to Ara h 8 (KU_A/l)	0.03 (0.01; 0.28)	0.03 (0.01; 1.93)	0.639
Specific IgE to Ara h 9 (KU_A/l)	0.01 (0.01; 0.02)	0.01 (0.01; 0.04)	1.0
Number of major peanut allergens bound by IgE	1.0 (1.0; 2.0)	2.0 (1.0; 3.0)	0.062
Peanut-specific IgG4 (µg/l)	180 (120; 405)	380 (120; 1100)	0.301
Ratio of peanut-specific IgG4 to IgE	21.63 (5.78; 161.26)	11.26 (3.15; 76.75)	0.218
Other food allergy - n (%)	26 (93%)	21 (100%)	0.500
Atopic eczema - n (%)	24 (86%)	16 (76%)	0.470
Asthma - n (%)	11 (39%)	8 (38%)	1.0
Allergic rhinitis - n (%)	15 (54%)	12 (57%)	1.0
Pollen allergy - n (%)	7 (25%)	7 (33%)	0.542
BAT parameters	Mild/Moderate (n=28)	Severe (n=21)	p value
%CD63+ Peanut 0.1	0.3 (0; 3.1)	1.0 (0.2; 3.1)	0.189
%CD63+ Peanut 1	1.9 (0.3; 9.0)	5.5 (1.7; 33.5)	**0.012**

%CD63+ Peanut 10	7.7 (2.1; 34.7)	34.5 (10.7; 70.6)	**0.024**
%CD63+ Peanut 100	18.2 (2.4; 44.3)	47.7 (12.5; 68.5)	**0.009**
%CD63+ Peanut 1,000	19.9 (3.3; 35.7)	33.8 (13.0; 56.9)	0.066
%CD63+ Peanut 10,000	26.8 (5.5; 45.1)	51.0 (34.4; 58.8)	**0.021**
Mean %CD63 Peanut 10-100	13.4 (2.3; 40.3)	37.6 (8.5; 65.7)	**0.029**
AUC CD63 Peanut	71.5 (26.3; 176.7)	148.0 (63.1; 234.9)	**0.031**
Maximal %CD63+ to peanut	31.05 (9.37; 58.18)	59.34 (26.07; 75.59)	**0.043**
%CD63+ peanut/anti-IgE	0.54 (0.14; 1.02)	1.10 (0.78; 1.46)	**0.003**
EC_{50} (ng/ml) - CD63	10 (1; 100)	10 (1; 10)	0.164
CD_{sens}	8.83 (1.25; 69.97)	29.24 (7.33; 86.94)	0.079

Table 5.19 Demographic, clinical and BAT parameters between severe and non-severe reactors classified according to the severity score adopted by Van der Zee et al[175].

Values are expressed as number (percentage) or median (interquartile range). Significant p values are highlighted in bold. CD_{sens} was calculated based on the CD63 dose-response curve. Numbers in the left column indicate the concentration of peanut extract in ng/ml. Abbreviations: SPT, skin prick test; AUC CD63, area under the dose-response curve considering the percentage of CD63-positive basophils.

Demographic and clinical features	Mild/Moderate (n=28)	Severe (n=21)	p value
Age (years)	5.3 (4.3; 5.8)	5.4 (4.9; 6.1)	0.379
Males - n (%)	18 (64%)	16 (76%)	0.533
Cumulative threshold dose of peanut protein (g)	0.10 (0.03; 1.62)	0.10 (0.03; 0.37)	0.885
SPT to peanut (mm)	9 (4; 10)	10 (8; 13)	**0.041**
Specific IgE to peanut (KU_A/l)	1.81 (0.38; 9.93)	25.30 (5.34; 98.70)	**0.003**
Specific IgE to Ara h 1 (KU_A/l)	0.07 (0.01; 0.18)	0.22 (0.10; 20.30)	**0.005**
Specific IgE to Ara h 2 (KU_A/l)	0.54 (0.09; 2.20)	11.0 (1.79; 54.30)	**0.001**
Specific IgE to Ara h 3 (KU_A/l)	0.03 (0.01; 0.04)	0.05 (0.01; 0.94)	0.111
Specific IgE to Ara h 8 (KU_A/l)	0.03 (0.01; 0.04)	0.03 (0.01; 1.93)	0.835
Specific IgE to Ara h 9 (KU_A/l)	0.01 (0.01; 0.02)	0.01 (0.01; 0.04)	1.0
Number of major peanut allergens bound by IgE	1.0 (0; 2.0)	2.0 (1.0; 3.0)	**0.006**
Peanut-specific IgG4 (µg/l)	160 (120; 443)	350 (123; 1043)	0.190
Ratio of peanut-specific IgG4 to IgE	34.15 (6.47; 167.78)	10.05 (2.52; 66.05)	0.087
Other food allergy - n (%)	26 (93%)	21 (100%)	0.500
Atopic eczema - n (%)	22 (79%)	18 (86%)	0.714
Asthma - n (%)	10 (36%)	9 (43%)	0.768
Allergic rhinitis - n (%)	14 (50%)	13 (62%)	0.563
Pollen allergy - n (%)	7 (25%)	7 (33%)	0.542
BAT parameters	Mild/Moderate (n=28)	Severe (n=21)	p value
%CD63+ Peanut 0.1	0.3 (0; 2.5)	1.0 (0.2; 3.2)	0.135
%CD63+ Peanut 1	1.9 (0.3; 5.1)	13.0 (1.7; 36.4)	**0.005**

%CD63+ Peanut 10	6.1 (2.1; 24.3)	39.6 (13.4; 71.3)	**0.002**
%CD63+ Peanut 100	13.3 (2.4; 41.8)	49.2 (17.1; 69.2)	**0.001**
%CD63+ Peanut 1,000	14.6 (3.3; 33.9)	35.2 (20.7; 58.2)	**0.023**
%CD63+ Peanut 10,000	23.7 (5.5; 45.1)	51.0 (30.7; 58.8)	**0.014**
Mean %CD63 Peanut 10-100	12.5 (2.3; 31.8)	45.0 (11.4; 69.1)	**0.003**
AUC CD63 Peanut	64.3 (26.3; 133.3)	170.3 (80.8; 258.4)	**0.005**
Maximal %CD63+ to peanut	26.80 (9.37; 50.60)	59.63 (32.44; 78.09)	0.008
%CD63+ peanut/anti-IgE	0.53 (0.14; 0.83)	1.29 (0.92; 1.54)	**<0.001**
EC_{50} (ng/ml) - CD63	10 (1; 100)	5.5 (1; 10)	**0.026**
CD_{sens}	4.83 (0.93; 23.93)	39.82 (12.12; 99.10)	**0.009**

Logistic regression analyses classifying the severity of allergic reactions according to the other severity scores and according to the need for adrenaline corroborated my findings using the severity classification adopted by Ewan *et al*[148] (Table 5.20), except for the logistic regression analyses using the classification by Brown *et al* where the p value for %CD63+ peanut/anti-IgE was above the cut-off for statistical significance (p=0.057).

Table 5.20 Results of multivariable analyses of factors associated with the severity of allergic reactions to peanut classified using different severity classification systems.

Significant p values are highlighted in bold.

Classification of severity	Variable	OR (95% CI)	p value
Mueller[172]	%CD63+ peanut/anti-IgE	0.121 (0.025; 0.592)	**0.002**
Sampson[174]	%CD63+ peanut/anti-IgE	0.062 (0.016; 0.244)	**0.009**
Van Der Zee[175]	%CD63+ peanut/anti-IgE	0.195 (0.031; 1.247)	**0.002**

5.7 Conclusions

Basophils are effector cells of food-induced anaphylaxis. Allergen-induced basophil reactivity and sensitivity *in vitro* indicates the severity and threshold of allergic reactions to peanut during oral peanut challenges, respectively. Basophil reactivity (assessed by the ratio of the percentage of CD63-positive basophils at 100 ng/ml of peanut to the percentage of CD63-positive basophils following stimulation with anti-IgE) is associated with severity and basophil sensitivity (measured by CD_{sens}) is associated with threshold of allergic reactions to peanut. Patients with a ratio of the percentage of CD63-positive basophils at 100 ng/ml of peanut to the percentage of CD63-positive basophils following stimulation with anti-IgE greater or equal than 1.3 were 3 times more likely to have severe allergic reactions to peanut. Patients with CD_{sens} greater or equal than 84.0 were 2 times more likely to react to low amounts of peanut. The basophil activation test may therefore be used as an *in vitro* surrogate for oral food challenges to estimate the severity and the threshold of allergic reactions and to improve the management of patients with peanut allergy.

Chapter 6 Additional applications of the basophil activation test

The basophil activation test (BAT) can have applications beyond the diagnosis of allergy and the characterisation of the immune response to allergens. During my PhD, I participated in collaborative projects with colleagues within King's College London in which I used the BAT to detect the biological activity of peanut protein in complex mixtures such as house dust extracts and to detect the ability of auto-antibodies present in the serum of asthmatic patients to induce basophil degranulation. I also investigated signalling pathways during peanut-induced basophil activation.

6.1 Determination of the biological activity of peanut protein in house dust

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Understanding how children become sensitised to peanut may lead to the development of strategies to prevent peanut allergy. Exposure to peanut can occur through various routes: *in utero*, via breast milk, via dietary gastrointestinal exposure or via environmental exposure through the skin or through inhalation. Epicutaneous exposure, particularly in children with abraded skin due to eczema, may play an important role in peanut sensitisation[88, 176]. Environmental exposure to peanut has been identified as a risk factor for the development of peanut allergy. Applying a food frequency questionnaire to children with peanut allergy, children with egg allergy who are at high-risk of developing peanut allergy and low-risk non-allergic controls and all their household members, Fox et al[145] demonstrated a dose-response relationship between the household peanut consumption, which is an indirect marker of environmental peanut exposure, and the risk of later development of peanut allergy. In a follow-on study, Brough et al[177] quantified the peanut protein in house dust samples using a polyclonal peanut ELISA and observed that peanut protein levels in the environment were positively correlated to peanut consumption in the home. Quantifying peanut protein in the home environment raised the question as to whether peanut allergens in house dust maintained their biological activity.

Functional *in vitro* assays that closely resemble *in vivo* allergic reactions have been used in previous studies to verify the allergenic potential of peanut proteins, i.e. their ability to trigger mast cell and basophil activation and degranulation, both pure and in complex mixtures[46, 178]. Mediators, such as β-hexosaminidase and histamine, released by rat basophilic leukaemia cells and stripped-human basophils passively sensitised with plasma from allergic patients have been measured following stimulation with allergen[179]. More recently, assays evaluating the expression of activation markers on the surface of basophils of allergic patients by flow cytometry, similar to the BAT described in the previous chapters, have been used to assess the allergenicity of foods[180] and have been shown to be a sensitive and specific tool to detect low amounts of peanut in food matrices[181]. I hypothesised that BAT would be useful in verifying the biological activity of peanut proteins in household dust samples.

6.1.1 Peanut protein in house dust is able to activate basophils from peanut allergic children

Three children mono-allergic to peanut and 3 non-allergic children were recruited to test the ability of peanut allergens contained in house dust samples to elicit basophil activation. Two of the patients with peanut allergy were sensitised to the 3 major peanut allergens (Ara h 1, Ara h 2, and Ara h 3), and 1 patient was sensitised to Ara h 1 and Ara h 2. None of these patients were sensitised to any other food or airborne allergens, as assessed by SPT. Extracted dust samples with high or low peanut protein content were tested using blood from children with peanut allergy and non-allergic children, forming 5 pairs of dust sample–patient experiments.

Dust samples containing peanut protein levels of less than 0.375 ng/ml did not induce significant basophil activation neither in children with peanut allergy (medians below 1% of CD63-positive basophils, Figure 6.1.A) nor in non-allergic children (medians below 2.7% of CD63-positive basophils, Figure 6.1. C). In contrast, dust samples containing high concentrations of peanut protein resulted in dose-dependent activation of basophils from peanut allergic (Figure 6.1.B) but not from non-allergic children (Figure 6.1.D). The proportion of CD63-positive basophils was significantly higher in children with peanut allergy at peanut concentrations ranging between 1 and 10,000 ng/ml ($p<0.01$) compared to non-allergic children.

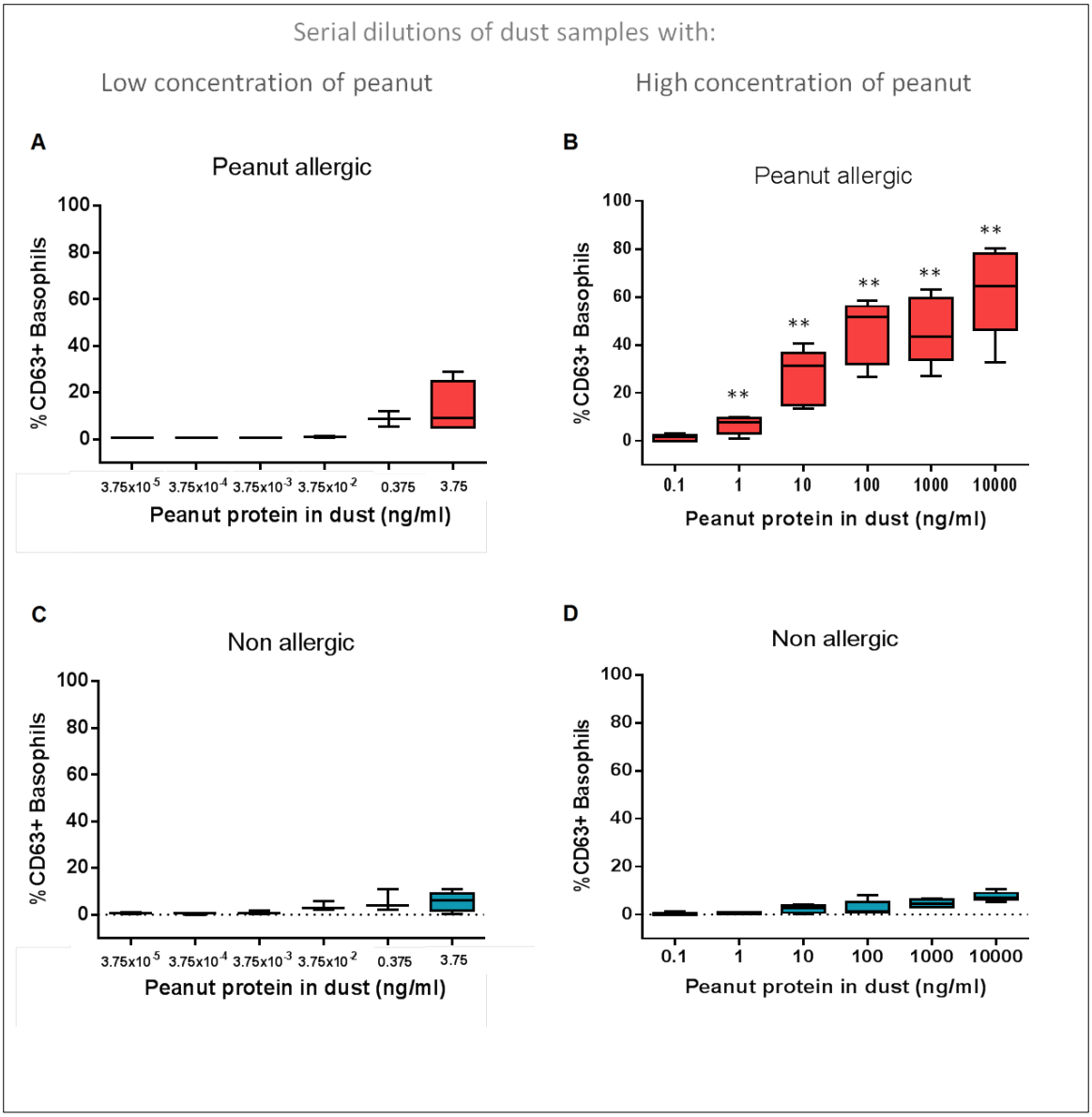

Figure 6.1 Activation of basophils from peanut mono-allergic or non-allergic patients by dust samples.

Activation of basophils was induced by serial dilutions of dust samples containing low (A and C) or high (B and D) peanut protein in peanut-monoallergic (A and B) or non-allergic (C and D) patients (n=5 pairs of sample-patient experiments). Quartiles and minimum and maximum percentages of CD63-positive basophils are displayed. High peanut dust extracts were dilution-adjusted to the maximum same concentration of peanut protein (10 µg/ml) and then underwent serial dilutions for comparability of dose-response, and low peanut dust samples were prepared by using the same dilution factor. Basophil activation for each concentration of peanut was compared between allergic and non-allergic children (i.e., A vs. C and B vs. D). **p<0.01.

The threshold for basophil activation was 1 ng/ml using basophils from the 3 selected donors with peanut allergy (Figure 6.1.B versus Figure 6.1.D). Basophils of allergic and non-allergic patients showed comparable spontaneous basophil activation (p=0.690) and CD63 expression induced by anti-IgE (p=0.222) and N-formyl-methionyl-leucyl-phenylalanine (p=0.841) - Table 6.1. In children with peanut allergy, the dose-response of basophil activation by dust samples was comparable with the dose-response to similar concentrations of the independent peanut standard. Basophils

of non-allergic patients were not activated by the peanut standard up to 10 µg/ml of peanut protein.

Table 6.1 Basophil activation of peanut allergic and non-allergic children

Basophil activation is expressed as median (interquartile range) of the percentage of CD63-positive basophils stimulated with high-peanut-containing dust samples or the peanut standard or controls. Abbreviations: RPMI, Roswell Park Memorial Institute medium (negative control); fMLP, Formyl-Methionyl-Leucyl-Phenylalanine.

Peanut protein (ng/ml)	Peanut allergic children		Non-allergic children	
	Dust samples	Peanut standard	Dust samples	Peanut standard
0.1	1.6 (0; 4.3)	2.0 (1.0; 25.1)	0.1 (-0.3; 0.8)	0 (0; 0.1)
1	7.8 (4.4; 9.6)	1.0 (0.62; 77.0)	0.6 (0.3; 0.9)	0.1 (0; 0.3)
10	31.2 (14; 36.6)	13.5 (9.1; 77.0)	2.8 (0.7; 3.7)	0.5 (0; 1.4)
100	51.7 (26.6; 56.1)	26.8 (20.1; 58.9)	1.3 (0.9; 5.1)	0 (0; 0.5)
1,000	43.3 (33.3; 59.5)	27.8 (27.4; 52.8)	4.6 (3.1; 6.2)	0.4 (0; 1.4)
10,000	64.4 (46.2; 78.2)	44.8 (38.8; 45.3)	7.2 (6.2; 9.0)	1.1 (0; 2.6)
Controls	Peanut allergic children		Non-allergic children	
RPMI	0.9 (0.87; 0.95)		1.5 (0.85; 1.75)	
Anti-IgE	22.0 (17.6; 63.7)		12.0 (9.6; 35.5)	
fMLP	35.6 (19.9; 62.3)		22.9 (3.9; 66.8)	

6.1.2 Conclusions

BAT is a useful tool in determining the allergenicity and the biological activity of peanut proteins, in complex mixtures, such as house dust. The peanut protein in the dust samples tested retained the ability to interact with immune cells, causing activation and degranulation of basophils from peanut allergic children. This suggests that peanut protein present in house dust may be an important source of allergenic protein that is biologically active and therefore could be implicated in allergic sensitisation. For example, peanut protein in house dust may be able to interact with dendritic cells present in the abraded and inflamed skin of patients with eczema leading to allergic sensitisation to peanut, which in turn can lead to peanut allergy.

6.2 Determination of the ability of auto-anti-IgE antibodies to activate peripheral blood basophils

Published in Chan YC, Ramadani F, Santos AF, et al. J Allergy Clin Immunol 2014; 134 (6): 1394-1401 (open-access).

Auto-antibodies, namely anti-IgE or anti-FcεRI, have been described in chronic urticaria[182], atopic eczema[183] and asthma[184]. By cross-linking IgE or aggregating FcεRI receptors, some of these auto-antibodies are able to activate mast cells and basophils and this activation is independent of allergens. Different methods have been used to measure the serum levels (e.g. ELISA-based methods) and to determine the function of such antibodies, including the autologous serum skin test[182], the basophil histamine release test[185] and, more recently, the basophil activation test using flow cytometry[186]. However, the levels of the auto-antibodies present in the serum do not necessarily reflect their inflammatory activity[187]. Furthermore, these auto-antibodies are also present in the serum of healthy individuals and may not have any pro-inflammatory activity.

Asthma develops in non-atopic individuals and exacerbations can happen independently of allergen exposure. One possible mechanism involved in the elicitation of basophil and mast cell degranulation that can initiate the exacerbation of asthmatic symptoms independently of allergen is the presence of auto-antibodies anti-IgE or anti-FcεRI. Two hypotheses were considered to explain the involvement of IgE in asthma, independent of allergen: 1. IgG anti-IgE antibodies were elevated in the serum of asthmatics irrespective of atopic status compared with controls; and 2. some IgG anti-IgE antibodies were able to activate mast cells and basophils whereas others had a regulatory role and inhibited allergen-induced effector cell activation.

6.2.1 Naturally occurring IgG anti-IgE antibodies are detectable in the serum of asthmatic patients

Serum samples of atopic asthmatics (AA), non-atopic asthmatics (NAA) and non-atopic non-asthmatic controls (NAC) were tested for IgG anti-IgE antibodies using an in-house custom ELISA using omalizumab as a standard to calibrate the ELISA[184]. Ten serum samples (6 from NAA and 4 from AA) showed concentrations of serum IgG anti-IgE above the upper limit of the 95% confidence interval of the measurements in NAC (Figure 6.2.). There were no significant differences in the levels of anti-IgE antibodies between the 3 groups of patients (Table 6.2).

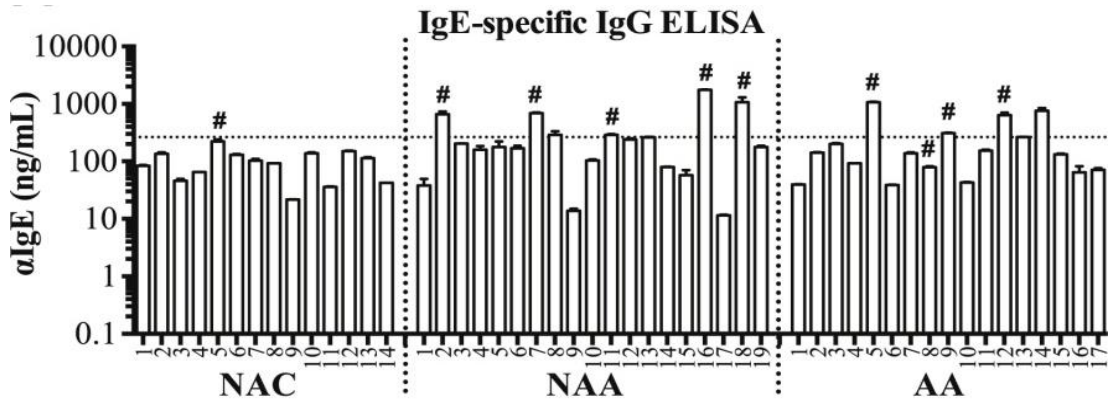

Figure 6.2 Concentrations of IgG anti-IgE autoantibodies in sera from non-atopic controls (NAC), non-atopic asthmatic subjects (NAA), and atopic asthmatic subjects (AA).

The dotted line shows 95% confidence limit of the range in controls. Samples marked # were used in later experiments.

Table 6.2 Concentration of IgG anti-IgE antibodies in the serum

Range is indicated per group.

	Non-atopic controls	Non-atopic asthmatics	Atopic asthmatics
Serum IgG anti-IgE antibodies (ng/ml)	22-223	11-1761	39-1070

6.2.2 Some serum samples containing IgG anti-IgE auto-antibodies induce basophil activation

The serum samples were also tested on a BAT. Basophils from an atopic donor were incubated with different test serum samples or with autologous serum or buffer (as negative controls) or with polyclonal goat anti-human IgE or monoclonal mouse anti-human FcεRI or fMLP (as positive controls) and basophil activation was assessed by flow cytometry (Figure 6.3, Figure 6.4, Figure 6.5).

Sera from 2 NAA (NAA17 and NAA18) and 3 AA (AA5, AA8, AA14) subjects were able to induce basophil activation. However, this activity did not equate with elevated IgG anti-IgE antibody concentration. For example, serum sample from AA8 activated basophils but the levels of auto-anti-IgE were below the defined reference line; conversely, serum samples from NAA2, NAA16 and AA12 had levels of auto-anti-IgE above the reference line but did not cause basophil activation.

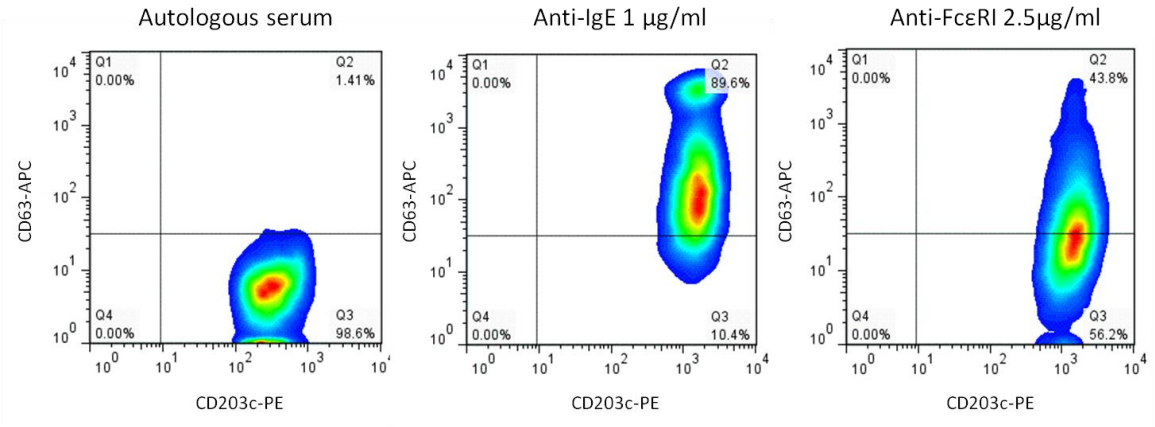

Figure 6.3 Basophil activation in response to the negative and to the positive controls.

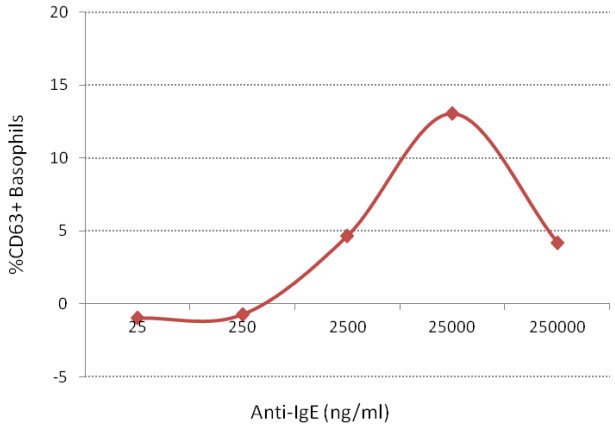

Figure 6.4 Basophil activation induced by serial dilutions of a serum sample of an asthmatic patient with elevated levels of IgG anti-IgE antibodies as determined by ELISA.

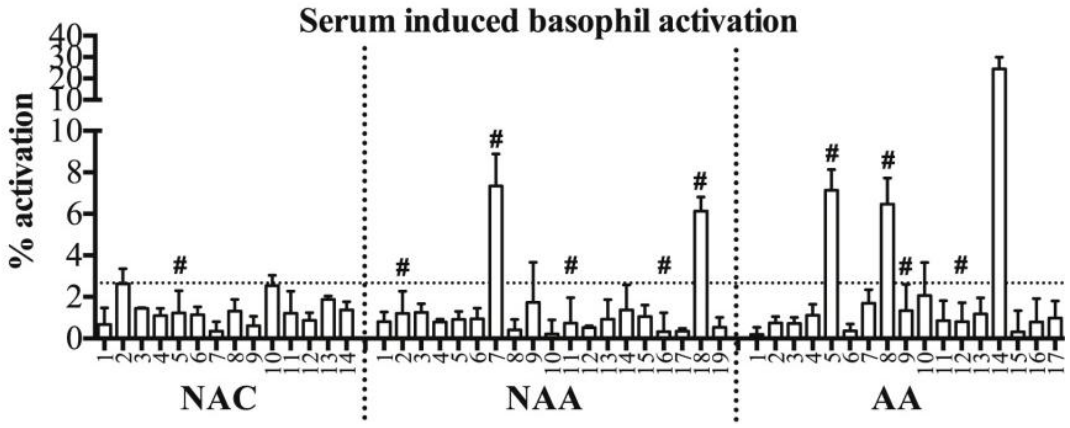

Figure 6.5 Basophil activation induced by serum samples of non-atopic controls (NAC), non-atopic asthmatics (NAA) and atopic asthmatics (AA).

The dotted line indicates the detection threshold (defined as 3 standard deviations above the mean percentage of CD63-positive basophils following incubation with autologous serum, i.e. >2.67% CD63-positive basophils). Samples marked # were used in later experiments.

6.2.3 IgG depletion from serum samples abolished the observed activatory effect

Depletion of IgG from the serum samples that were able to cause basophil activation reduced this effect, confirming that basophil activation was due to the presence of IgG antibodies (p=0.041, Figure 6.6).

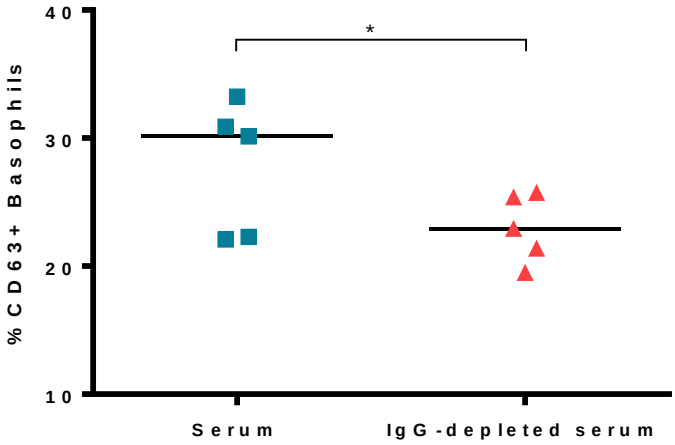

Figure 6.6 Serum samples causing basophil activation were depleted of IgG and re-tested on the basophil activation assay.

Basophil activation was reduced following incubation with IgG-depleted samples (n=5), *p<0.05.

6.2.4 IgG anti-IgE antibodies modify allergen-induced basophil activation by inhibiting the allergen-IgE interaction

To further examine the effect of serum samples on allergen-induced basophil activation, PBMCs from the same donor used in previous experiments were incubated with the serum samples, anti-IgE or autologous serum and then stimulated with house dust mite allergen Der p 2. Serum samples from two asthmatic subjects (NAA16 and AA12) inhibited Der p 2-induced basophil activation. Depletion of IgG (Figure 6.7. A) or IgE-binding proteins (Figure 6.7. B) abolished this effect and purification of IgE-binding proteins retained the inhibitory activity (Figure 6.7.C and D).

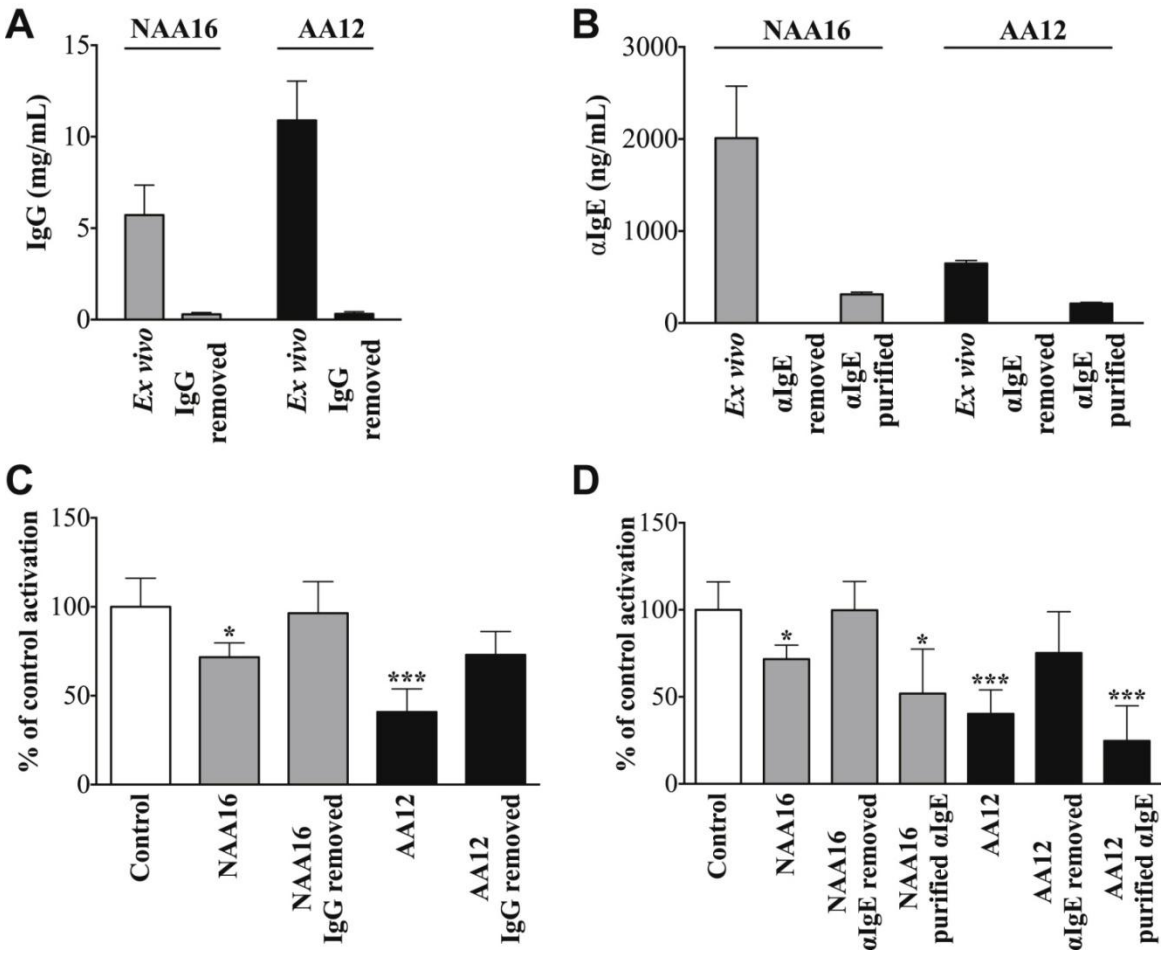

Figure 6.7 Antibody levels and basophil activation following IgG depletion and depletion or purification of IgE-binding proteins.

A. Total IgG concentration in 2 test sera *ex vivo* and following IgG depletion. B. IgE-binding proteins ex vivo and following depletion and isolation of IgE binding proteins. C and D. Effects of sera *ex-vivo* and following IgG depletion (C) and removal and purification of IgE binding proteins (D) on Der p 2-induced basophil activation induced by Der p 2 (30 ng/ml). Bars represent the mean/standard deviation of 3 independent experiments. *p<0.05 and ***p<0.001.

To confirm the inhibitory activity of the sera, cells from the basophilic cell line RBL-SX38 were sensitised with recombinant IgE specific for the grass allergen Phl p 7 and stimulated with recombinant Phl p 7. Pre-incubation with serum samples from NAA16 or AA12 significantly reduced binding of recombinant allergen to IgE but not of allergen-specific IgE to RBL-SX38 cells, indicating that IgG anti-IgE antibodies modify allergen-induced basophil activation by inhibiting the allergen-IgE interaction (Figure 6.8).

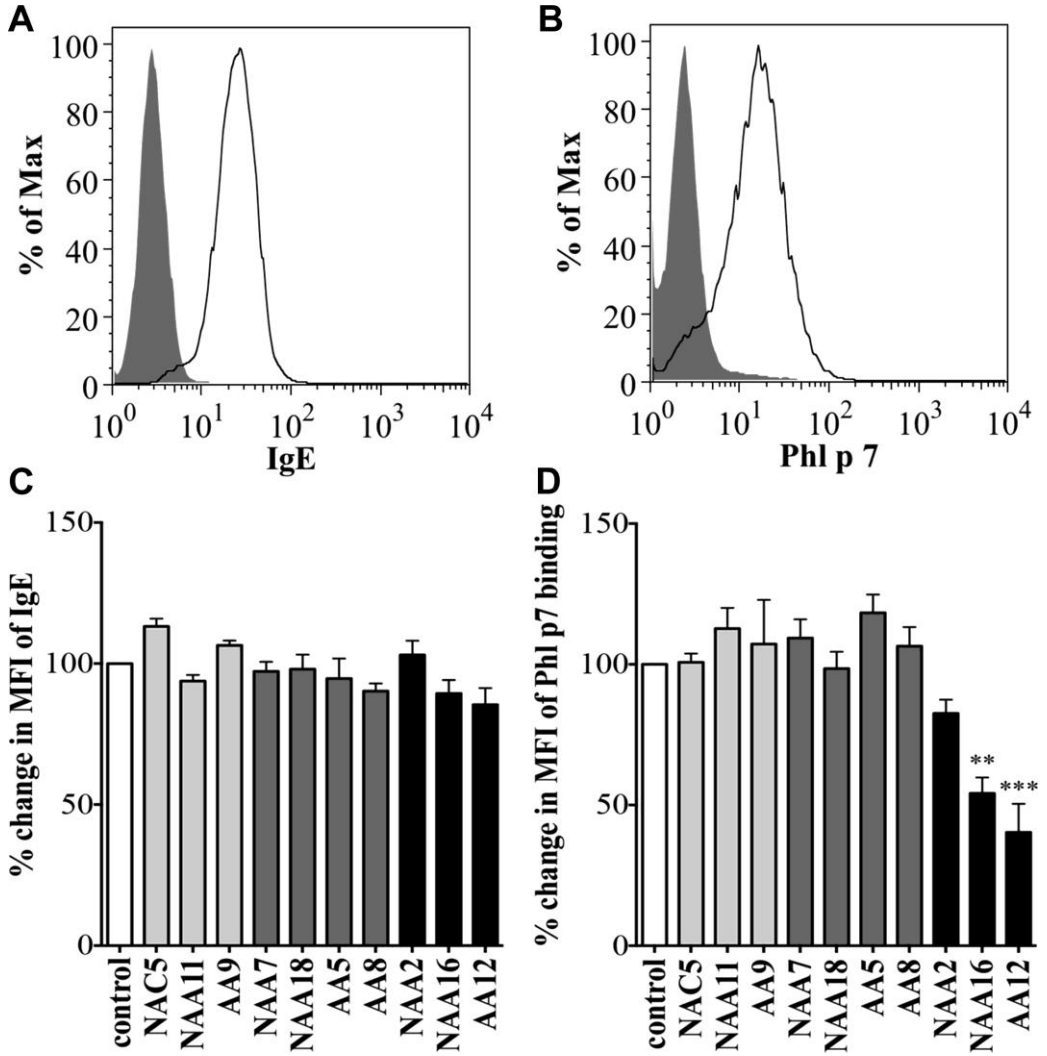

Figure 6.8 Modification of allergen-induced basophil activation by anti-IgE antibodies

A. Binding of recombinant Phl p 7-specific IgE to FcεRI on RBL-SX38 cells. B. Binding of Phl p 7 to IgE-sensitised RBL-SX38 cells. C and D. Changes in binding of Phl p 7-specific IgE (C) and changes in binding of Phl p 7 (D) on RBL-SX38 cells sensitised with Phl p 7-specific recombinant IgE and pre-incubated with sera, followed by stimulation with recombinant Phl p 7 compared with no serum control. Bars represent the mean/standard deviation of 3 independent experiments. **p<0.01 and ***p<0.001.

6.2.5 Conclusions

IgE-specific auto-antibodies were detectable in atopic and non-atopic asthmatic subjects and controls. They bound to IgE regardless of whether or not IgE was bound to its high-affinity receptor. Some of these auto-antibodies activated basophils, whereas others inhibited allergen-induced basophil activation. Activatory anti-IgE auto-antibodies could cause clinical symptoms of asthma independently of allergen exposure and could explain some phenotypes of asthma classified as non-atopic. Inhibitory IgG anti-IgE auto-antibodies may contribute to a natural regulatory mechanism that could conceivably influence the severity and presence or absence of clinical expression of IgE-mediated diseases, including asthma and food allergy, and the

outcomes of therapeutic processes such as allergen immunotherapy and therapy with exogenous IgG anti-IgE.

6.3 Basophil intracellular signalling

Cross-linking of IgE molecules bound to FcεRI by allergens initiates a cascade of intracellular events downstream of the high affinity IgE receptor leading to basophil degranulation, including phosphorylation of intracellular signalling molecules, both activatory (such as Syk and p38MAPK) and inhibitory (such as SHP and SHIP) - Figure 1.2.

I aimed to determine the patterns of phosphorylation of some of these intracellular signalling molecules that accompany the expression of basophil activation markers and to confirm that phosphorylation of some of these intracellular signalling molecules and the expression of activation markers on the surface of basophils can be simultaneously assessed by flow cytometry.

6.3.1 Up-regulation of CD63 is accompanied by the phosphorylation of Syk and p38 MAPK

Stimulation of basophils from peanut allergic patients with peanut extract resulted in the phosphorylation of Syk and p38-MAPK (Figure 6.9)

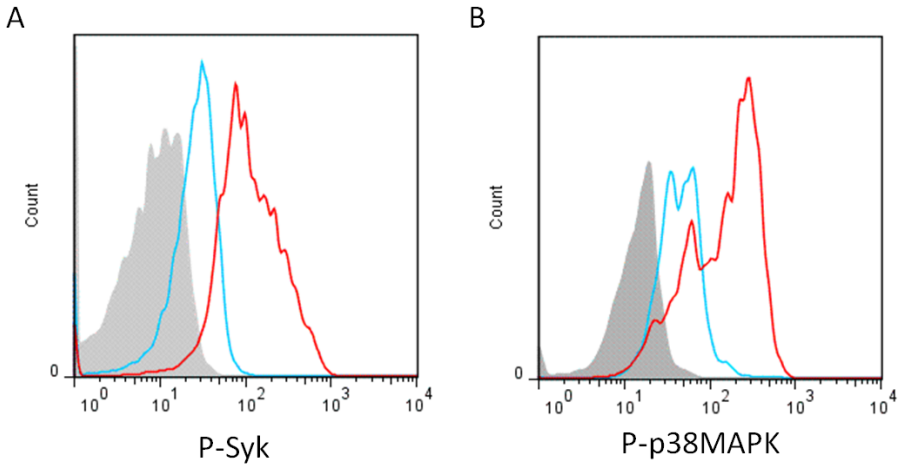

Figure 6.9 Peanut-induced phosphorilation of Syk and p38-MAPK

Stimulation of basophils with 1000ng/ml of peanut extract resulted in up-regulation of phosphorylated Syk (A) and phosphorylated p38-MAPK (B). Histograms represent the isotype control (grey), the negative control (blue) and cells stimulated with 1000 ng/ml of peanut extract (red).

A time course was performed to identify the kinetics of phosphorylation of these two activatory signalling molecules. The maximum expression of phosphorylated Syk and p38MAPK was detected at 3 minutes (Figure 6.10.A and B). The phosphorylation of these signalling mediators preceded the up-regulation of the activation marker CD63 (Figure 6.10.E).

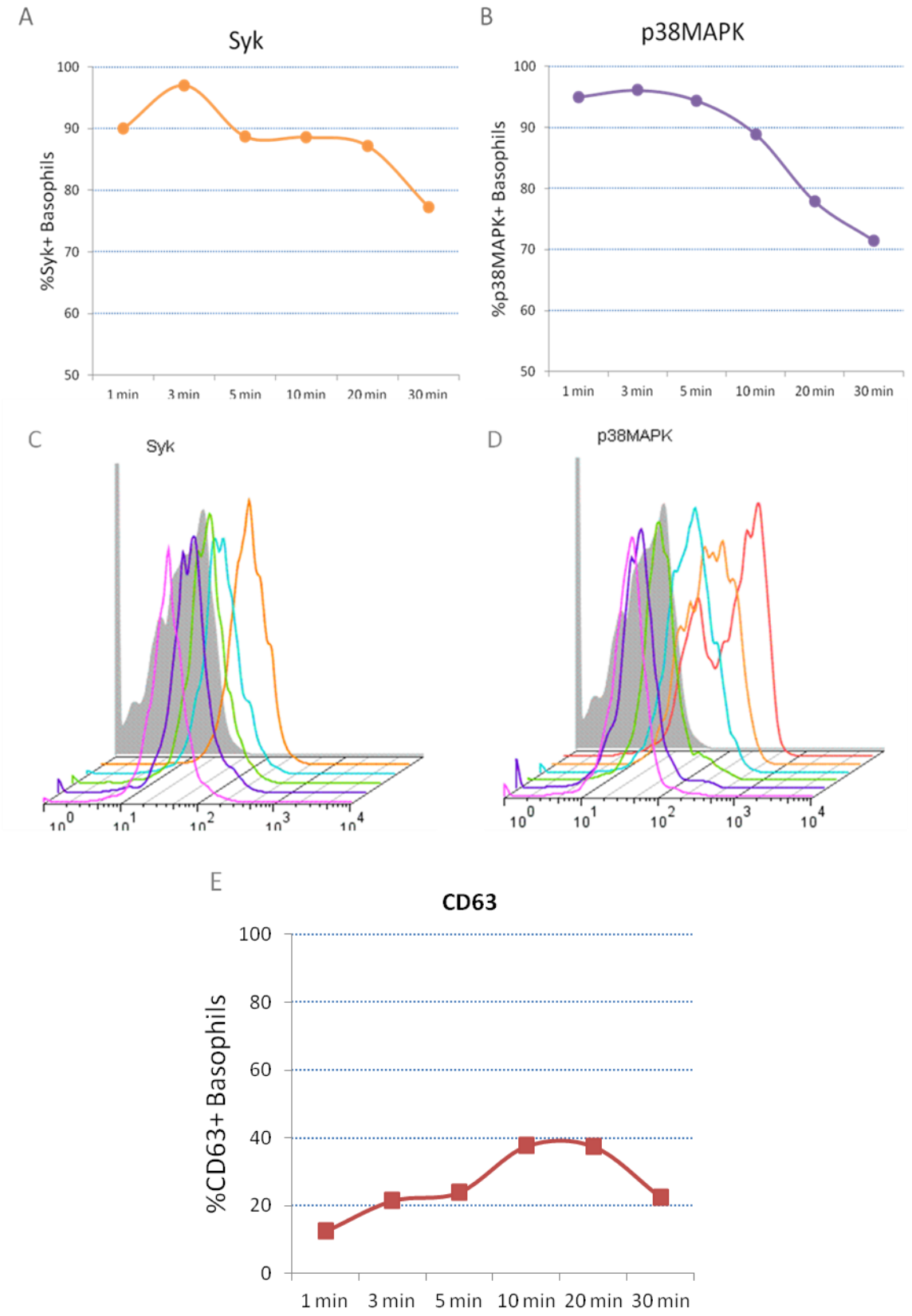

Figure 6.10 Peanut-induced expression of phosphorylated Syk, phosphorylated p38MAPK and CD63 at different time-points.

Expression of phosphorylated Syk (A, C), phosphorylated p38MAPK (B, D) and CD63 (E) following stimulation with 1000 ng/ml of peanut extract at different time points. Figures C and D represent histograms of MFI for P-Syk-PE and P-p38-MAPK-PE at the various time-points studied: 1 minute (light violet), 3 min (dark violet), 5 minutes (green), 10 minutes (blue), 20 minutes (orange) and 30 minutes (red).

Phosphorylation of p38MAPK and up-regulation of CD63 developed in the same proportion of cells, suggesting that these two phenomena happened in the same basophil population (Figure 6.11).

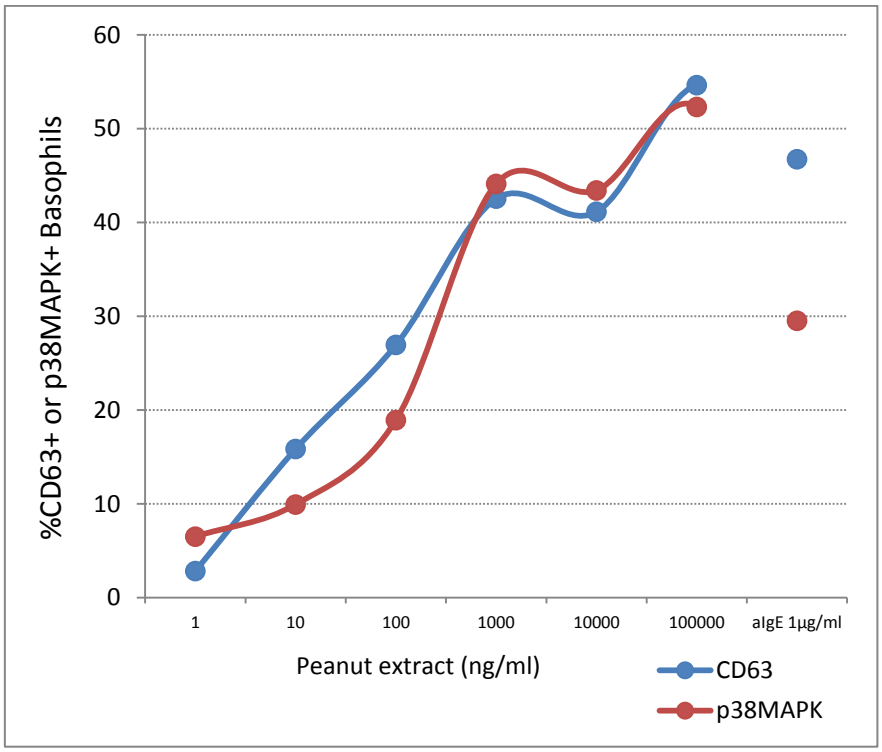

Figure 6.11 Expression of CD63 on the cell surface and of phosphorylated p38MAPK intracellularly in basophils of a peanut allergic patient after stimulation with 1000ng/ml of peanut extract for 30 minutes.

6.3.2 Conclusions

Intracellular and surface markers can be independently and simultaneously assessed by flow cytometry. Combined analysis of basophil intracellular signalling molecules and activation markers may complement studies on immune mechanisms involving basophils. A kinetic analysis of basophil signalling showed that Syk and p38-MAPK phosphorylation occurred early with a maximum expression at 3 minutes following stimulation with allergen and preceded the up-regulation of activation markers. This corresponds to the signalling pathway downstream of FcεRI (Figure 1.2), leading to basophil activation with CD63 up-regulation and mediator release.

Chapter 7 Disentangling the discrepancy between clinical allergy and sensitisation to peanut

Published in Santos AF et al. IgG4 inhibits peanut-induced basophil and mast cell activation in peanut tolerant children sensitized to peanut major allergens. J Allergy Clin Immunol 2015 (in press, open-access).

The discrepancy between allergic sensitisation and clinical food allergy creates diagnostic difficulties and a fundamental gap in our knowledge about the mechanisms of food allergy and tolerance. I have used peanut allergy as a model of IgE-mediated food allergy. Specific IgE to Ara h 2 is particularly discriminative in identifying PA patients[43, 44]; however, examples can be found of peanut tolerant patients who have high levels of Ara h 2-specific IgE and conversely of PA patients who test negative to Ara h 2 and the other major peanut allergens[188].

Basophils and mast cells are effector cells of acute allergic reactions to foods, including anaphylaxis. As reported in chapter 4, the whole blood BAT has high accuracy in the diagnosis of peanut allergy and correlates very closely with the clinical phenotype of IgE-sensitised patients, i.e. allergic versus tolerant patients, better than specific IgE levels to peanut, to Ara h 2 or to any of the other peanut components[165]. Similar *in vitro* systems using passive sensitisation of basophils or mast cells with patients' plasma can be used to test the ability of allergen-specific IgE antibodies present in the plasma to elicit effector cell activation and degranulation in response to allergen.

Two non-mutually exclusive hypotheses were addressed to explain the discrepancy between allergic sensitisation and clinical allergy to peanut, as described in Section 1.13. The first hypothesis was that the levels and the specificity of IgE are different between allergic and tolerant patients. The second hypothesis was that sensitised but tolerant patients have an inhibitor that blocks the function of IgE. Given that natural tolerance to peanut is allergen-specific and long-lasting, the IgE inhibitor is likely to be a food-specific antibody of an isotype other than IgE, such as IgG4. IgG4 has been shown to increase in patients who naturally outgrow IgE-mediated food allergy, such as cow's milk allergy[102, 103], and in patients who are submitted to immunotherapy to foods[110, 112] or to immunotherapy to respiratory allergens[108, 109]. Whether IgG4 can play an inhibitory role in the allergen-IgE interaction in sensitised but otherwise tolerant patients is unknown. IgG4 is produced as part of a Th2-type immune response, induced mainly by the

tolerogenic cytokine IL-10[144], and was therefore the main suspect of being the IgE inhibitor in PS patients.

7.1 IgG4 study population

Two hundred and twenty eight children, 108 peanut allergic and 110 peanut tolerant (77 PS and 43 NA), were included in this analysis. PS patients included 6 children (3% of total and 8% of PS) who had outgrown peanut allergy. PA children were slightly older (median 6 years-old) and more frequently had asthma (39.8%) and allergic rhinitis (59.3%) than PS children (median 4 years-old, 18.2% with asthma and 31.2% with allergic rhinitis). The other demographic and clinical features were similar between the two groups (Table 7.1).

Table 7.1 Demographic and clinical features of the study population

N=228. Number (percentage) or median (interquartile range) are represented. *p values refer to the comparison between peanut allergic and peanut sensitised but tolerant patients.

Demographic and clinical characteristics	Peanut allergic (n=108)	Peanut tolerant (n=110)		p value*
		Peanut-sensitised but tolerant (n=77)	Non-peanut sensitised non-allergic (n=43)	
Age (years)	6.0 (5.0; 10.0)	4.0 (1.5; 7.5)	5 (5.0; 7.0)	<0.001
Males - n (%)	74 (68.5%)	47 (61.0%)	30 (69.8%)	0.185
Other food allergy - n (%)	94 (87.0%)	67 (87.0%)	9 (20.9%)	0.582
Atopic eczema - n (%)	71 (65.7%)	46 (59.7%)	18 (41.9%)	0.248
Asthma - n (%)	43 (39.8%)	14 (18.2%)	8 (18.6%)	0.001
Allergic rhinitis - n (%)	64 (59.3%)	24 (31.2%)	12 (27.9%)	<0.001
Pollen allergy - n (%)	51 (47.2%)	25 (32.5%)	9 (20.9%)	0.075
Non atopic - n (%)	0 (0%)	0 (0%)	14 (32.6%)	-

In parallel, samples from an independent population of 19 peanut allergic patients that had been submitted to peanut oral immunotherapy (POIT) at Addenbroke's Hospital in Cambridge, as part

of the STOP I trial, were tested. Samples were collected before and between 6 to 24 months after treatment (Table 7.2).

Table 7.2 Time to follow-up and peanut protein tolerated before and after peanut oral immunotherapy in the participants in the STOP I trial.

Abbreviations: ID, identification; OIT, oral immunotherapy.

Subject ID	Peanut protein tolerated pre-OIT (g)	Time to follow up at post-OIT visit	Peanut protein tolerated post-OIT (g)
POIT1	0.200	24 months	0.80
POIT2	0.031	24 months	0.80
POIT3	0.556	24 months	0.80
POIT4	0.256	12 months	6.46
POIT5	0.006	12 months	0.83
POIT6	0.006	24 months	0.80
POIT7	0.031	18 months	0.80
POIT8	0.081	18 months	0.80
POIT9	0.006	24 months	0.80
POIT10	0.081	18 months	0.80
POIT11	0.256	24 months	0.80
POIT12	0.001	12 months	6.25
POIT13	0.001	12 months	6.57
POIT14	0.006	6 months	2.49
POIT15	0.256	24 months	0.80
POIT16	0.006	24 months	0.80
POIT17	0.001	6 months	0.025
POIT18	0.031	12 months	~ 5.6
POIT19	0.110	6 months	2.50

7.2 Passive sensitisation basophil and mast cell assays reproduced *in vitro* patients' clinical reactivity to peanut

As described in chapter 4, the whole blood basophil activation test to peanut reproduced very closely the clinical phenotype (i.e. peanut allergic or peanut tolerant) of peanut-sensitised patients[161]. Similar experiments were performed in which LAD2 cells or IgE-stripped primary basophils from healthy donors were sensitised with plasma from PA, PS or NA patients prior to stimulation with peanut extract. Mast cells sensitised with plasma from PA patients, but not from PS or NA patients, showed a dose-dependent activation in response to peanut. Figure 7.1 represents the activation of LAD2 cells sensitised with plasma from PA, PS or NA patients, and stimulated with peanut extract, expressed as percentage of CD63-positive cells. The same findings were found for the percentage of CD107a+ LAD2 cells and with primary human basophils (Figure 7.2).

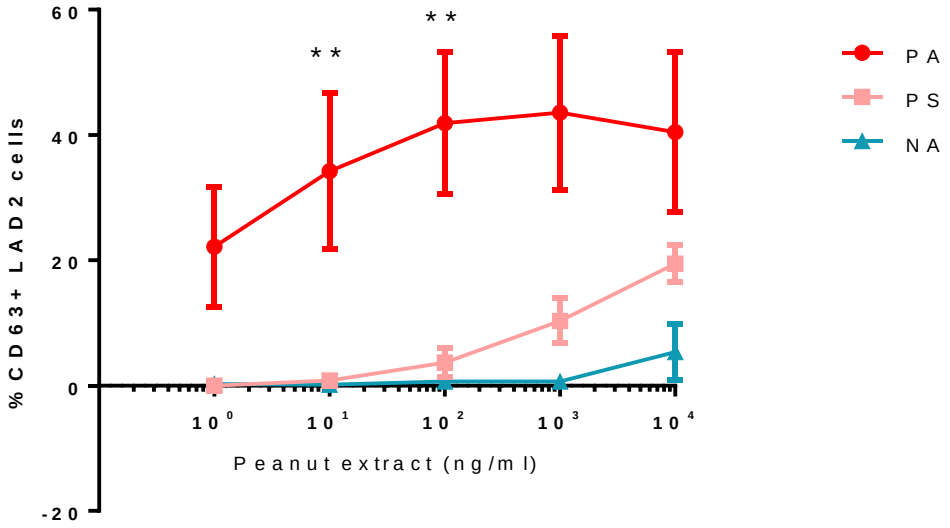

Figure 7.1 Peanut-induced activation of mast cells sensitised with plasma from peanut allergic, peanut-sensitised but tolerant and non-sensitised non-allergic patients.

PA (n=6), PS (n=5), NA (n=2). Mean and standard error are represented. p value refers to the comparison between PA and PS for each concentration of peanut extract. **p<0.01

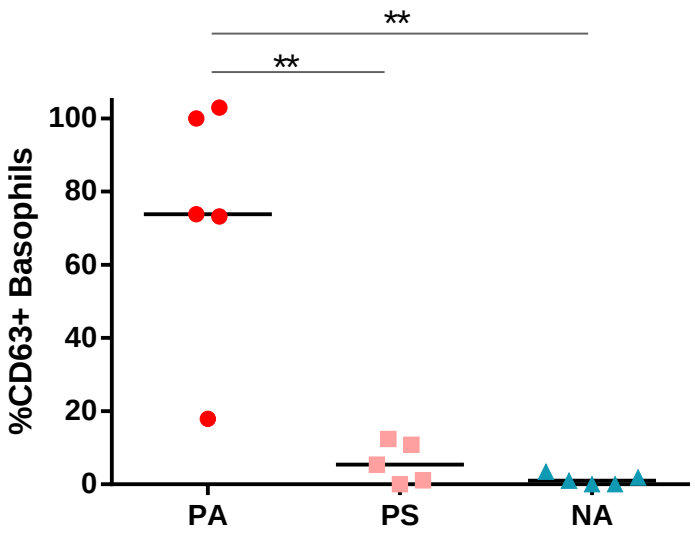

Figure 7.2 Peanut-induced activation of basophils sensitised with plasma from peanut allergic, peanut-sensitised but tolerant and non-sensitised non-allergic patients

N=15, 5 per group. Abbreviations: PA, peanut allergic; PS, peanut-sensitised but tolerant; NA, non-sensitised non-allergic. LAD2 cells were stimulated with 10 ng/ml of peanut extract. To correct for the variability in cell reactivity among basophil donors, the percentage of CD63-positive basophils is expressed as the ratio between the maximal reactivity to peanut extract with tested plasma and the maximal reactivity to peanut extract with plasma from a peanut allergic subject used as an internal control in all experiments. Horizontal lines represent the median for each group. p values correspond to the comparison between PA and PS patients and between PA and NA patients. The comparison between PS and NA groups was non-significant **p<0.01.

7.3 Differences in peanut-specific IgE between peanut allergic and peanut sensitised but tolerant individuals

7.3.1 Levels of specific IgE to peanut and to peanut components only partially explained differences in clinical reactivity to peanut between peanut allergic and peanut sensitised but tolerant individuals

PA patients showed higher levels of specific IgE to peanut (p<0.001) and to Ara h 1 (p<0.001), Ara h 2 (p<0.001) and Ara h 8 (p=0.019) than PS patients (Table 7.3). However, there was a substantial overlap between the 2 groups, as illustrated in Figure 7.3.

Table 7.3 Skin prick test to peanut and serum specific IgE levels to peanut and to peanut components.

N=228, except if otherwise indicated.[*]Median (interquartile range) are indicated. p values refer to the comparison between peanut allergic and peanut sensitised but tolerant patients. Significant p values are highlighted in bold. [x]n=222; [+]n=221; [¬]n=220; [#]n=219.

		Peanut allergic (n=108)	Peanut tolerant (n=110)		p value*
			Peanut-sensitised but tolerant (n=77)	Non-peanut sensitised non-allergic (n=43)	
Skin prick test to peanut (mm)		9 (7; 12)	4 (1; 8)	0 (0; 0)	**<0.001**
Specific IgE (KU_A/l)	Peanut	13.30 (2.26; 98.70)	1.83 (0.48; 5.20)	0.01 (0.01; 0.02)	**<0.001**
	Ara h 1[x]	0.44 (0.04; 31.13)	0.11 (0.01; 0.34)	0.01 (0.01; 0.01)	**<0.001**
	Ara h 2[x]	6.04 (0.90; 54.55)	0.08 (0.03; 0.25)	0.01 (0.01; 0.04)	**<0.001**
	Ara h 3[+]	0.12 (0.02; 2.41)	0.06 (0.02; 0.34)	0.01 (0.01; 0.01)	0.075
	Ara h 8[¬]	0.08 (0.01; 2.09)	0.01 (0.01; 0.23)	0.01 (0.01; 0.01)	**0.019**
	Ara h 9[#]	0.01 (0.01; 0.08)	0.02 (0.01; 0.25)	0.01 (0.01; 0.01)	0.169

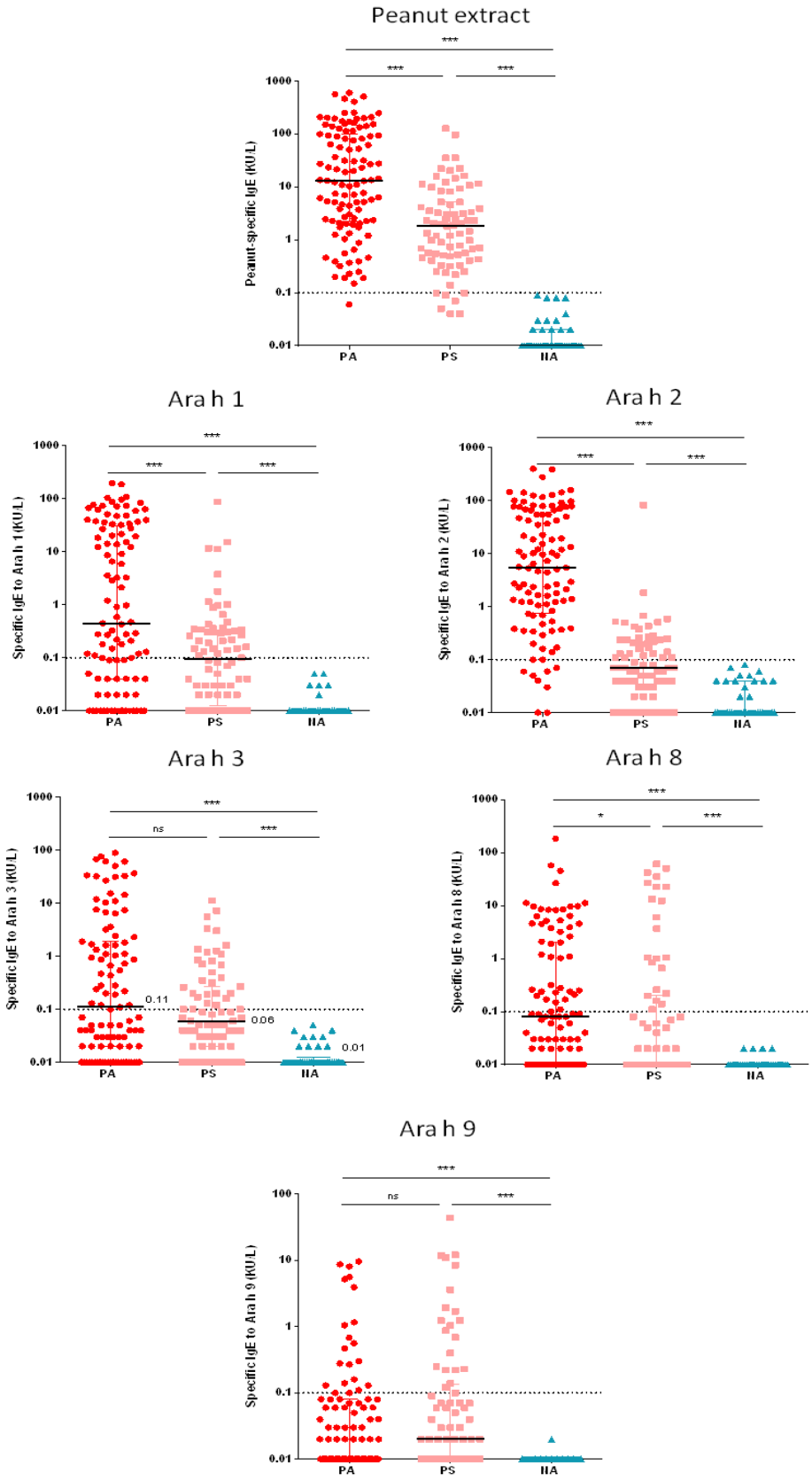

Figure 7.3 Specific IgE to peanut and to peanut components in peanut allergic, peanut sensitised but tolerant and non-sensitised non-allergic children.

N=228 for peanut-specific IgE; n=222 for specific IgE to Ara h 1 and Ara h 2; n=221 for specific IgE to Ara h 3; n=220 for specific IgE to Ara h 8; n=219 for specific IgE to Ara h 9. Horizontal lines represent the median for each group. Abbreviations: PA, peanut allergic; PS, peanut-sensitised but tolerant; NA, non-sensitised non-allergic. *p<0.05 ***p<0.001.

7.3.2 Profiles of IgE sensitisation to peanut allergens did not distinguish all cases of peanut allergic and peanut-sensitised but tolerant patients

There was no obvious relationship between the profiles of IgE sensitisation to peanut allergens and clinical reactivity (Table 7.4). The majority (67.8%) of PA patients were sensitised to more than one peanut allergen (Ara h 1, Ara h 2 and/or Ara h 3) whereas PS patients were more likely to be sensitised to one or none of the peanut major allergens (60.5%). Although there was a substantial overlap between PA and PS patients, there were two unique patterns of sensitisation, one per group: all patients sensitised simultaneously to Ara h 1 and to Ara h 2 were PA and all patients monosensitised to Ara h 1 were PS. Table 7.5 shows the prevalence of sensitisation to the five tested peanut allergens.

Table 7.4 Profiles of IgE sensitisation to major peanut allergens in peanut allergic and in peanut-sensitised but tolerant children.

Only patients with IgE results for all peanut components were included. N=103 PA and 76 PS. The percentage of patients per group is represented. Specific IgE ≥ 0.10 KU_A/l were considered a positive result. Significant p values are highlighted in bold.

Profiles of IgE sensitisation	Peanut allergic (%)	Peanut-sensitised but tolerant (%)	p value
Ara h 1 2 & 3	48.5	23.7	**0.001**
Ara h 1 & 2	15.5	0	**<0.001**
Ara h 2 & 3	1.9	3.9	0.355
Ara h 1 & 3	1.9	11.8	**0.007**
Ara h 1	0	15.8	**<0.001**
Ara h 2	26.9	21.1	0.234
Ara h 3	1.0	3.9	0.203
No Ara h 1 2 3	3.8	19.7	**0.001**

Table 7.5 Prevalence of sensitisation to the peanut components in peanut allergic and peanut-sensitised but tolerant patients.

Only patients with IgE results for all peanut components were included. N=103 PA and 76 PS. Sensitisation was defined as a level of specific IgE ≥0.10 KU_A/l. The number and percentage of patients per group are represented. Significant p values are highlighted in bold.

Prevalence of sensitisation	Peanut allergic	Peanut-sensitised but tolerant	p value
Ara h 1	69 (64%)	39 (51%)	0.096
Ara h 2	97 (90%)	37 (48%)	**<0.001**
Ara h 3	55 (51%)	33 (43%)	0.299
Ara h 8	46 (43%)	23 (30%)	0.091
Ara h 9	23 (21%)	25 (33%)	0.092

7.4 High ratio of peanut-specific IgG4 to IgE was associated with peanut tolerance

7.4.1 Peanut-specific IgG4 was higher in peanut-sensitised but tolerant patients compared to peanut allergic patients

One hundred and one patients (65 PA, 27 PS, 9 NA) with detectable peanut-specific IgG4 were randomly selected to be tested for IgG4 to individual peanut components. Considering this group of 101 patients, the levels of peanut-specific IgG4 were 1.6-fold higher in PS than in PA patients (p=0.012, Figure 7.4 and Table 7.6). However, there were no significant differences between the levels of specific IgG4 to peanut components in PA and PS patients (Figure 7.4 and Table 7.6), except for Ara h 2-specific IgG4 that was higher in PA patients (p=0.034).

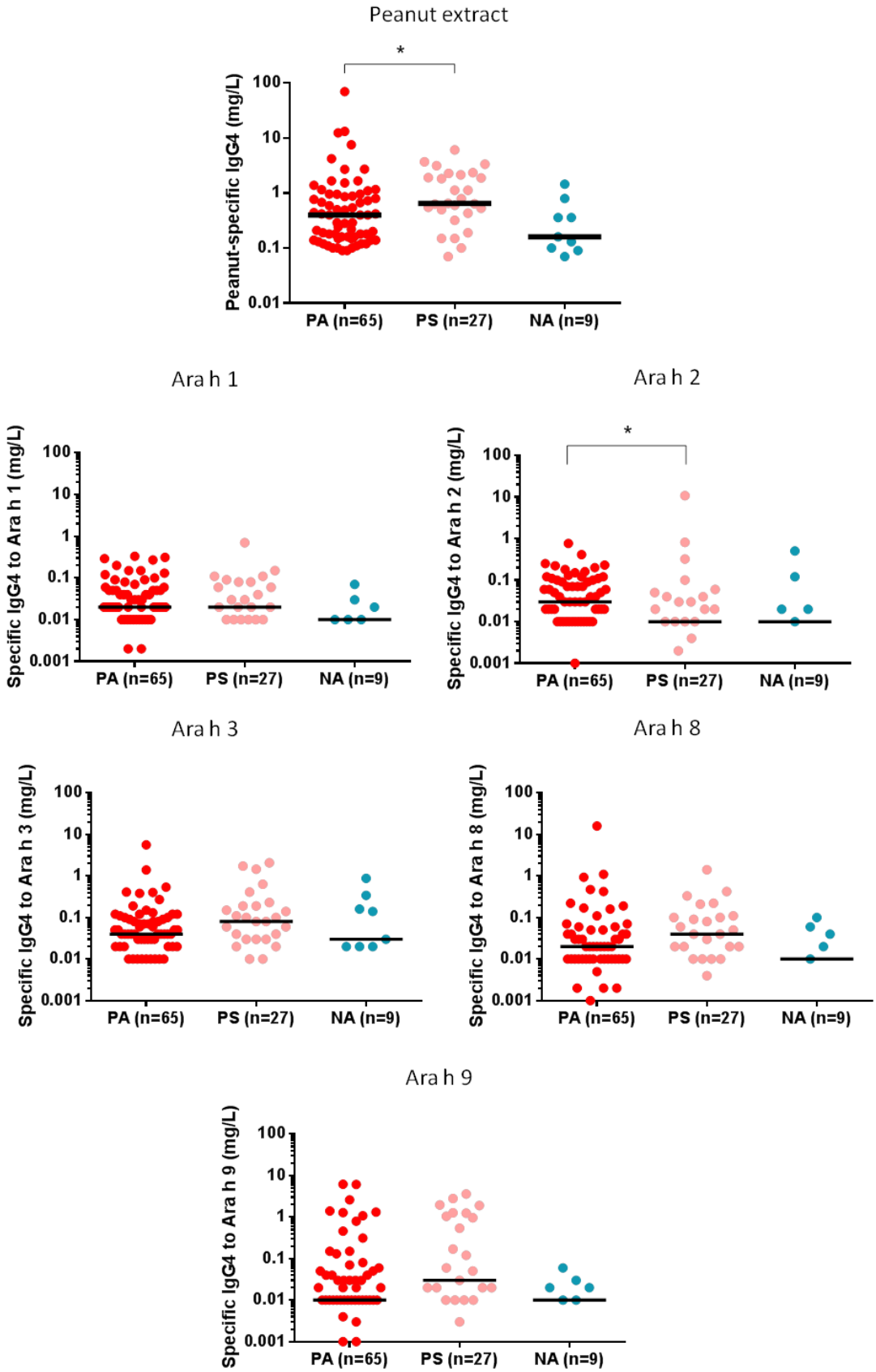

Figure 7.4 Specific IgG4 to peanut and peanut components in peanut allergic and peanut tolerant children, including peanut sensitised but tolerant and non-sensitised non-allergic children

N=101. Horizontal lines represent the median for each group. p values that were significant are indicated. Abbreviations: PA, peanut allergic; PS, peanut-sensitised but tolerant; NA, non-sensitised non-allergic. *p<0.05.

Table 7.6 Serum specific IgG4 levels to peanut and to peanut components in peanut allergic and peanut tolerant patients.

N=101. Median and interquartile range are represented. *p value refers to the comparison between peanut allergic and peanut sensitised but tolerant patients. Significant p values are highlighted in bold.

Peanut-specific IgG4 (µg/l)	Peanut allergic (n=65)	Peanut tolerant (n=36)		p value*
		Peanut-sensitised but tolerant (n=27)	Non-peanut sensitised non-allergic (n=9)	
Peanut	400 (160; 960)	650 (430; 2140)	160 (100; 580)	**0.023**
Ara h 1	20 (10; 60)	20 (10; 80)	10 (0; 30)	0.684
Ara h 2	30 (10; 100)	10 (0; 40)	10 (0; 70)	**0.034**
Ara h 3	40 (20; 100)	80 (30; 190)	30 (20; 250)	0.074
Ara h 8	20 (0; 50)	40 (10; 100)	10 (0; 50)	0.068
Ara h 9	10 (0; 60)	30 (10; 1040)	10 (0; 20)	0.065

The levels of peanut-specific IgA were also measured in the serum of 205 patients (105 PA, 66 PS and 34 NA) where sample was available. Peanut-specific IgA was detectable (≥1000 µg/l) in only 35 (17%) of these patients (28 PA, 5 PS and 2 NA) and ranged between 1.04 mg/l and 42.2 mg/l. The comparison between PA and PS did not reveal statistically significant differences (p=0.903 - Table 7.7).

Table 7.7 Serum peanut-specific IgA levels in peanut allergic, peanut sensitised but tolerant and non-sensitised non-allergic patients with detectable serum peanut-specific IgA.

Median and interquartile range are represented. Abbreviations: PA, peanut allergic; PS, peanut-sensitised but tolerant; NA, non-sensitised non-allergic. *p value corresponds to the comparison between PA and PS.

	PA (n=28)	PS (n=5)	NA (n=2)	p value*
Peanut-specific IgA (mg/l)	1.42 (1.04; 42.20)	1.36 (1.20; 2.87)	1.19 (1.08;1.30)	0.903

7.4.2 The ratio of peanut-specific IgG4 to IgE was significantly higher in peanut-sensitised but tolerant patients

Overall, the levels of specific IgG4 to peanut and to peanut components could not explain the clinical differences between PA and PS patients. I hypothesised that the relative amounts rather than absolute amounts of IgE and IgG4 could explain the differences in clinical phenotype and determined the ratios of IgG4 to IgE to peanut and to Ara h 1, Ara h 2, Ara h 3, Ara h 8 and Ara h 9. The ratio of specific IgG4 to IgE directed to peanut was 8 times higher in PS compared to PA patients (p<0.001, Figure 7.5). The differences between PS and PA patients were even greater in the ratio of IgG4 to IgE to the major peanut allergens, namely Ara h 1 (18.8-fold, p=0.05), Ara h 2 (100-fold, p=0.004) and Ara h 3 (7-fold, p=0.016).

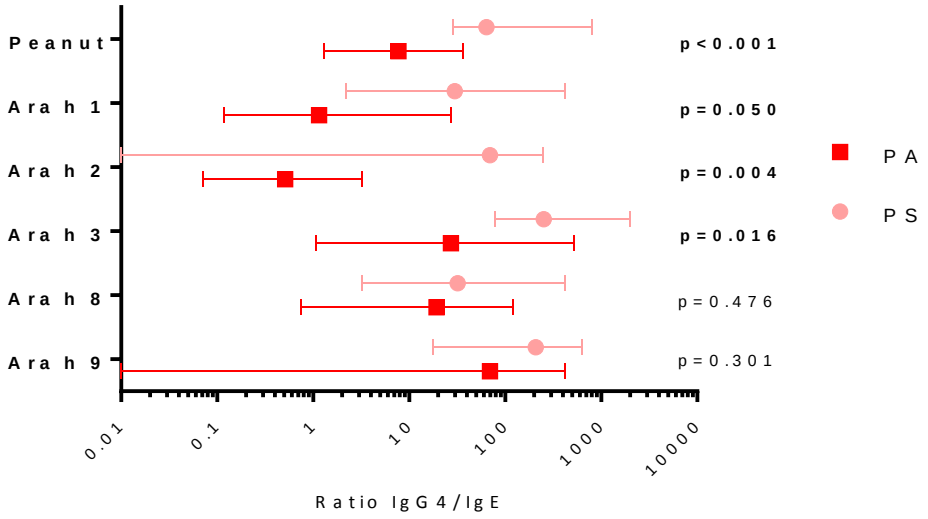

Figure 7.5 Ratio of IgG4 to IgE to peanut and to peanut allergens in peanut allergic and peanut sensitised but tolerant children.

N=65 PA and 27 PS. Median and interquartile range are represented. Abbreviations: PA, peanut allergic; PS, peanut-sensitised but tolerant. Significant p values are highlighted in bold.

The observed differences in the ratio of peanut-specific IgG4 to IgE between PA and PS patients could be due to differences in IgE rather than differences in the ratio itself. After adjusting for specific IgE levels by analysis of covariance using ranks, the differences in the ratio of peanut-specific IgG4 to IgE between PA and PS patients remained significant (p=0.001). Similarly, using a multivariate logistic regression model, log base 10 transformed IgG4 (p=0.004) and IgE (p<0.001) were both significantly associated with peanut allergy. A relative importance analysis showed that IgE accounted for 64% of the models explanatory power in predicting PA versus PS

patients, and IgG4 explained the remaining 36%. PA patients tended to have higher levels of IgE to peanut and to peanut major allergens whereas PS patients showed a predominance of IgG4 over IgE (Figure 7.6).

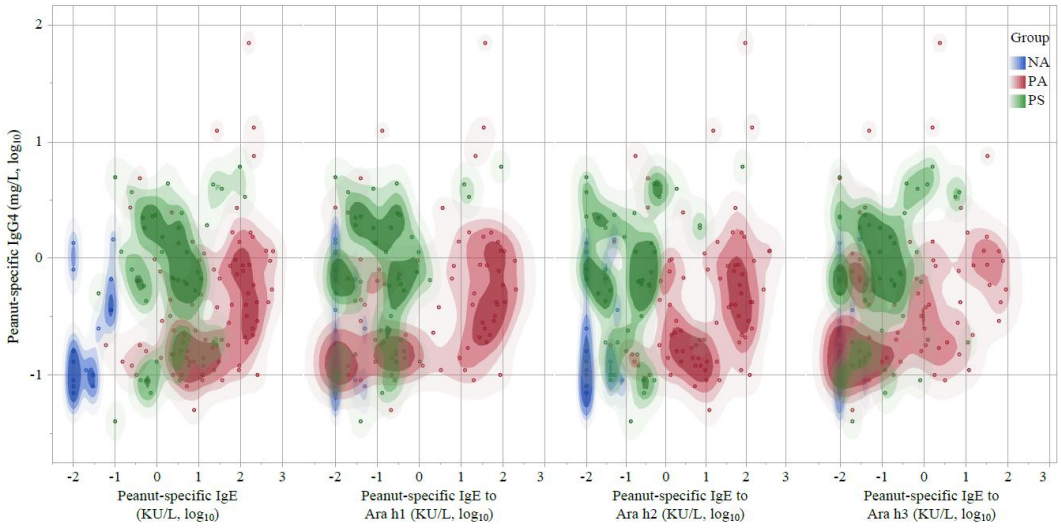

Figure 7.6 Distribution of IgE and IgG4 levels in peanut allergic, peanut sensitised but tolerant and non-sensitised non-allergic children.

N=101. Log base 10 of IgE and IgG4 levels are represented. Abbreviations: PA, peanut allergic; PS, peanut-sensitised but tolerant; NA, non-sensitised non-allergic.

In PA patients submitted to POIT (n=19), the levels of peanut-specific IgE were similar before and after treatment but the levels of peanut-specific IgG4 and the ratio of peanut-specific IgG4 to IgE were significantly higher post-immunotherapy (Table 7.8).

Table 7.8 Levels of specific IgE and IgG4 to peanut in peanut allergic patients submitted to peanut oral immunotherapy before and after treatment.

N=19. Median and interquartile range are represented. Significant p values are highlighted in bold.

	Pre-POIT	Post-OIT	p value
Peanut-specific IgE (KU_A/l)	27.30 (5.71; 100.0)	10.50 (2.83; 85.90)	p=0.573
Peanut-specific IgG4 (µg/l)	580 (270; 1510)	7200 (1210; 21200)	**p<0.001**
Ratio IgG4/IgE	16.77 (4.12; 96.80)	170.64 (30.95; 585.75)	**p=0.001**

7.4.3 Peanut-specific IgG4/IgE ratio was inversely correlated with basophil activation in response to peanut

In order to assess whether the ratio of IgG4 to IgE had a functional consequence in the ability of peanut extract to elicit basophil activation *in vitro*, the subgroup of patients for whom peanut-specific IgG4 had been determined and for whom whole blood basophil activation test to peanut had been performed were selected. Patients with non-responder basophils and patients with IgE below 0.10 KU_A/l, were excluded. The peanut-specific IgG4 to IgE ratio was inversely correlated with basophil activation in response to peanut using Spearman correlation coefficient (Rs=-0.686, p<0.001, n=55) - Figure 7.8.

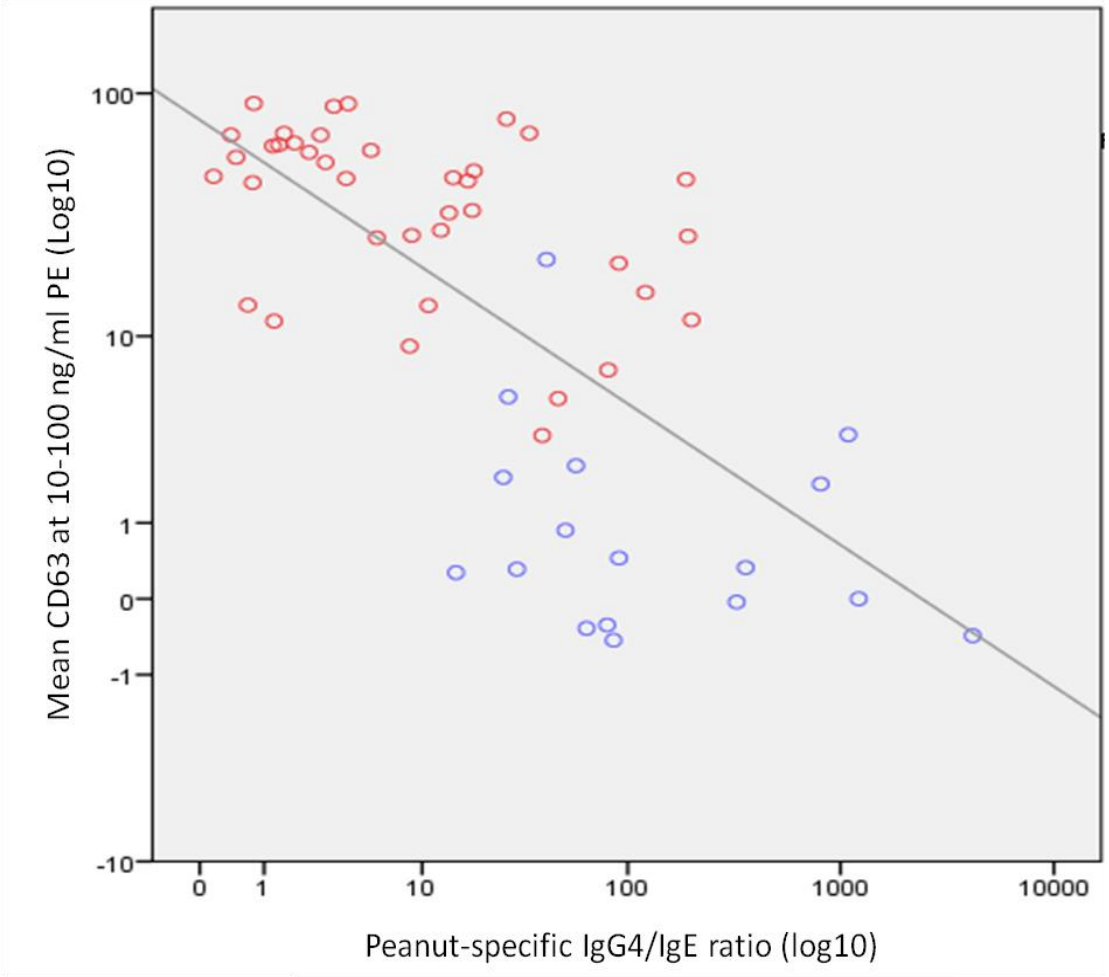

Figure 7.7 Peanut-specific IgG4/IgE ratio was inversely correlated with basophil activation in response to peanut.

Red circles represent peanut allergic patients and blue circles represent peanut sensitised but tolerant patients (n=55). Variables are represented in a logaritmic scale base 10. Basophil activation is expressed as the mean percentage of CD63-positive basophils at 10 and 100 ng/ml of peanut extract (PE).

7.5 IgG4 inhibits peanut-induced basophil and mast cell activation in peanut tolerant children sensitised to peanut major allergens

7.5.1 Plasma from peanut-sensitised tolerant (but not from non-sensitised non-allergic) patients were able to inhibit peanut-induced basophil and mast cell activation similar to plasma of peanut allergic patients submitted to peanut oral immunotherapy

The observations described in the previous section 7.4. suggested that IgG4 was likely to have an inhibitory role over IgE. I hypothesized that this would be most relevant to PS patients with IgE directed to the major peanut allergens Ara h 1, Ara h 2 and Ara h 3 that would otherwise be predictive of peanut allergy. The ability of plasma from these patients to inhibit mast cells and basophils sensitised with plasma from a reference PA patient also sensitised to Ara h 1, Ara h 2 and Ara h 3 was tested (Table 7.9). Samples from patients submitted to POIT (Table 7.8) and from NA patients (with and without detectable serum peanut-specific IgG4) were used as controls.

Table 7.9 Profiles of sensitisation of the peanut-sensitised but tolerant patients selected for the inhibition studies and of the peanut allergic patients used as a reference to assess inhibition of mast cell activation.

*These PS patients were also tested in the mast cell activation assay shown in Figure 7.1.

Participant ID	Specific IgE (KU_A/l)						Specific IgG4 (µg/l)					
	Peanut	Ara h 1	Ara h 2	Ara h 3	Ara h 8	Ara h 9	Peanut	Ara h 1	Ara h 2	Ara h 3	Ara h 8	Ara h 9
PS1	9.31	0.88	0.42	0.27	0.22	11.9	760	30	20	40	40	3570
PS2*	97.1	88.30	82.30	1.61	0.20	43.90	6090	700	10700	60	4	1040
PS3*	128	15.20	0.67	5.62	0.26	0.25	3360	80	30	1450	20	20
PS4*	35.7	0.08	1.84	0.71	1.01	8.48	3940	80	60	110	90	2770
PS5	5.21	0.36	0.24	0.2	0.01	11	600	90	40	80	50	1900
PS6*	5.18	0.96	0.52	0.68	0.01	0.02	1130	0	810	410	0	0
PS7*	3.16	1.79	0.26	0.17	6.06	0.22	650	40	10	100	80	960
PS8	0.71	0.13	0.28	0.12	0.08	0.1	70	0	0	0	0	0
PS9	2.09	0.21	0.24	0.32	0.85	0.02	320	0	0	10	0	10
PS10	22.2	11.7	0.59	1.21	0.01	0.01	4300	480	4720	620	0	0
PS11	3.95	0.3	0.16	0.35	62.3	0.22	2440	130	30	1410	2740	10
PS12	0.49	0.19	0.38	0.2	0.16	0.23	580	10	120	60	0	30
PA	255	84.4	125	1.8	0	0	100	30	20	10	0	10

Peanut-induced mast cell and basophil activation was inhibited in the presence of plasma from PS patients with IgE directed to the peanut major allergens, Ara h 1, Ara h 2 and Ara h 3. The results using LAD2 cells or primary human basophils that had been treated with lactic acid to remove IgE before sensitisation with the reference plasma sample from the peanut allergic patient were comparable (Figure 7.8). One sample of a peanut allergic patient was used as a reference for the mast cell assays and another sample of a peanut allergic patient (also with high levels of peanut-specific IgE and IgE to Ara h 1, Ara h 2, Ara h 3, Ara h 8 and Ara h 9) was used for the basophil assays (see Section 2.3.5.5. and Section 2.3.6.2.).

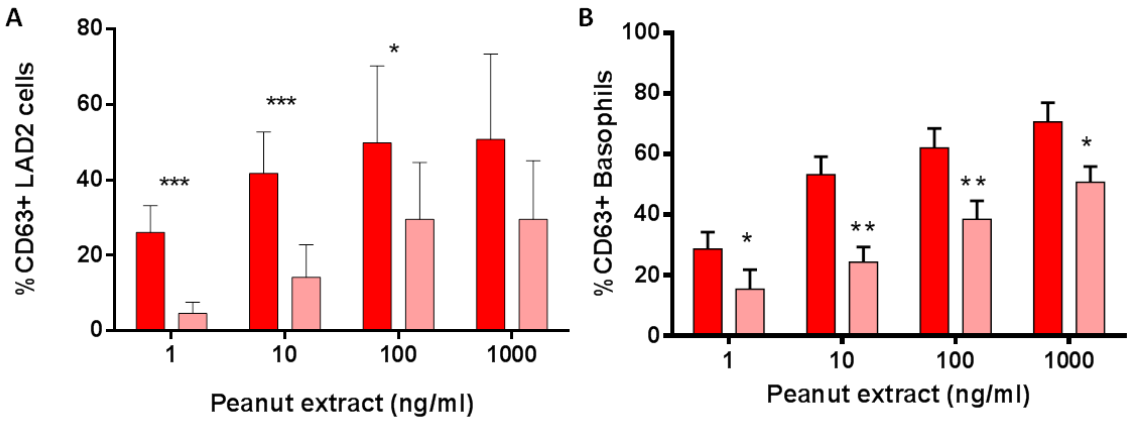

Figure 7.8 Plasma from peanut-sensitised but tolerant patients inhibits peanut-induced activation of mast cells and basophils sensitised with plasma from a peanut allergic patient.

Peanut-induced activation of mast cells (A) and basophils (B) sensitised with plasma from a peanut allergic patient (red) in the presence of plasma from peanut-sensitised but tolerant patients (pink, n=7 in A and n=9 in B). p values correspond to the comparison between groups for each concentration of peanut extract. *p<0.05 **p<0.01 ***p<0.001.

Samples of peanut allergic patients submitted to peanut oral immunotherapy were also able to inhibit peanut-induced activation of mast cells and basophils previously sensitised with a reference plasma sample from a peanut allergic patient or with plasma from the peanut allergic patients submitted to POIT collected before starting treatment (Figure 7.9).

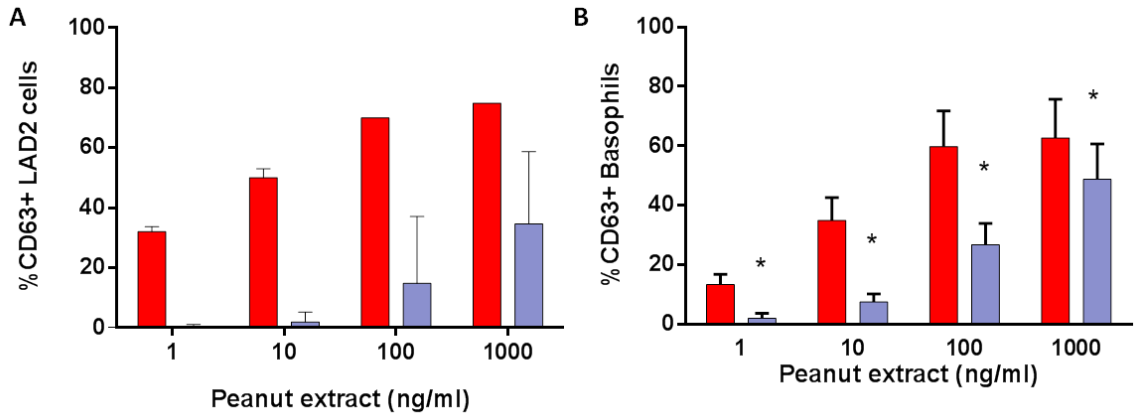

Figure 7.9 Plasma from peanut allergic patients collected post-oral immunotherapy inhibit peanut-induced activation of mast cells and basophils sensitised with plasma from a reference peanut allergic patient or plasma from peanut allergic patients collected pre-oral immunotherapy.

Peanut-induced activation of mast cells (A) and basophils (B) sensitised with a reference plasma from a peanut allergic patient (A) or with plasma samples of peanut allergic patients submitted to POIT collected before the start of the treatment (B) is inhibited in the presence of post-treatment plasma from patients who underwent peanut oral immunotherapy (A, n=3 and B, n=5). Red bars refer to activation of cells sensitised with plasma from a reference peanut allergic patient (A) or plasma from peanut allergic patients before POIT (B) and purple bars refer to activation of the cells sensitised with plasma from a reference peanut allergic patient (A) or plasma from peanut allergic patients before POIT (B) in the presence of plasma from peanut allergic patients submitted to POIT. In B, pairs of plasma samples from the same peanut allergic patient before and after POIT were used in each experiment. p values correspond to the comparison between groups for each concentration of peanut extract. *p<0.05.

Plasma samples of non-sensitised non-allergic patients were not inhibitory, regardless of whether they had detectable peanut-specific IgG4. Once again, the results of passive sensitisation experiments using the mast cell line and primary human basophils were comparable (Figure 7.10).

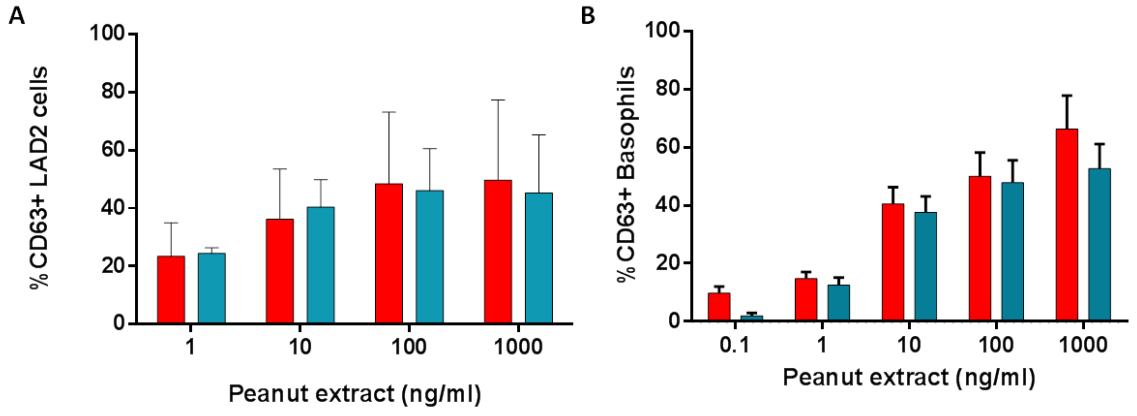

Figure 7.10 Plasma from non-sensitised non-allergic patients does not inhibit peanut-induced activation of mast cells and basophils sensitised with plasma from a peanut allergic patient.

Peanut-induced activation of mast cells (A) and basophils (B) sensitised with plasma from a peanut allergic patient (red) in the presence of plasma from non-sensitised non-allergic patients (blue) N=3 in A and n=3 in B. The differences between groups for each concentration of peanut extract were not statistically significant.

In summary, peanut-induced activation of mast cells and basophils sensitised with a reference plasma sample from a peanut allergic patient was inhibited in the presence of plasma from PS patients with IgE to the major peanut allergens and in the presence of plasma from patients submitted to POIT but not in the presence of plasma from NA patients (Figure 7.11.).

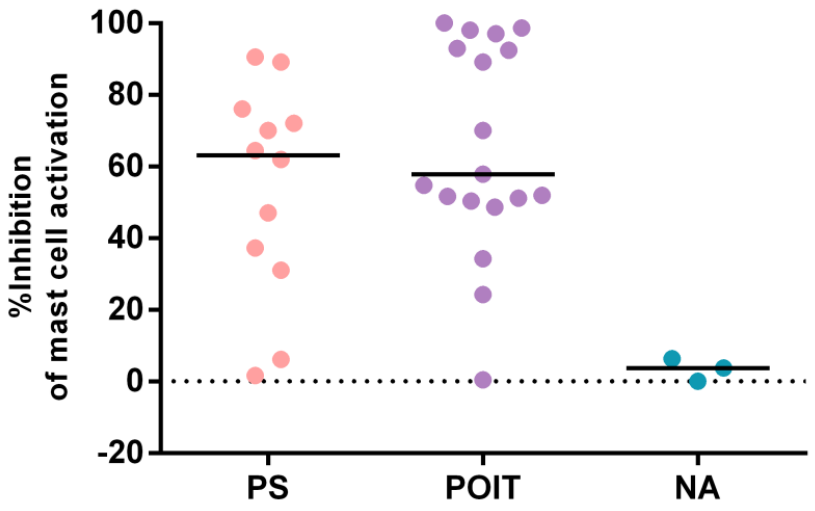

Figure 7.11 Inhibition of peanut-induced mast cell activation in the presence of plasma from peanut-sensitised but tolerant patients, from patients submitted to peanut oral immunotherapy and from non-sensitised non-allergic patients.

PS, n=12; POIT, n=19; NA, n=3. Cells were stimulated with 10 ng/ml of peanut extract. Horizontal lines represent the median for each group of patients. Inhibition was tested against the same plasma of a peanut allergic patient. %Inhibition = (%CD63+ of cells sensitised with PA plasma-%CD63+ of cells sensitised with PA plasma in presence of test plasma)/%CD63+ of cells sensitised with PA plasma.

The basophil and mast cell inhibition experiments were performed in the presence of plasma to assess the inhibitory effect of plasma components. The hypothesized inhibitory mechanisms may happen at the extracellular cellular level by competition with IgE for binding to the allergen and/or at the cellular level by co-cross-linking with IgE. If both mechanisms co-exist a correlation with symptoms is expected both in the presence of plasma and in plasma-free conditions; although inhibition may possibly be higher in the presence of plasma where the two mechanisms may occur concomitantly. Figure 7.12 represents the results of passive sensitisation experiments in which the inhibitory activity of the plasma was assessed in the presence of plasma and in plasma-free conditions, i.e. with and without a washing step following sensitisation with plasma from PS or NA patients and before stimulation with peanut extract. In these experiments, receptor-bound IgE was stripped of basophils from a healthy atopic donor (non-sensitised and non-allergic to peanut). These stripped basophils were subsequently sensitised with plasma from a PA patient.

Sensitised cells were then washed and sensitised with plasma from a PS patient (pink) or with plasma from a NA patient (blue) or with buffer (red), before stimulation with peanut extract or the positive controls or the negative control. As expected, untreated basophils from the healthy donor were not activated by peanut extract but, when stripped of IgE and sensitised with plasma from a PA patient, did show marked activation after peanut stimulation. The addition of PS plasma to the basophils previously sensitised with PA plasma markedly inhibited activation in response to peanut stimulation (Figure 7.12.A); however, this inhibition did not occur if PS plasma was washed before peanut stimulation (Figure 7.12.B). These results support the role of a component in the plasma of PS patients that is able to reduce peanut-induced basophil activation. The effect of a possible intracellular mechanism resulting from co-cross linking of IgE and an inhibitory antibody is not apparent.

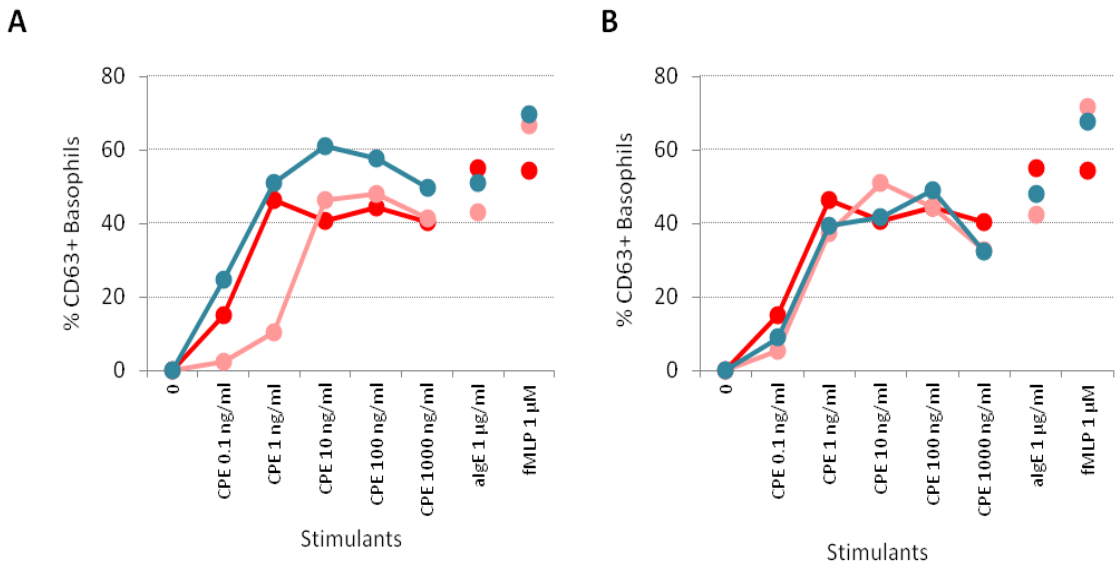

Figure 7.12. Modification of peanut-induced activation of basophils sensitised with plasma from a peanut allergic patient by plasma from peanut-sensitised but tolerant patient.

IgE was stripped of basophils from a healthy atopic donor (non-peanut allergic, no peanut-specific IgE) and the basophils were sensitised with plasma from a peanut allergic patient. Subsequently, plasma from a peanut sensitised but tolerant patient (pink) or from a non-sensitised non-allergic patient (blue) or media (red) were added to the basophils before stimulation with peanut extract or the positive controls (figure 7.12.A). In figure 7.12.B, the same steps were taken; however, basophils were washed before stimulation with the peanut extract. Abbreviations: CPE, crude peanut extract; aIgE, anti-IgE; fMLP, formyl-methionyl-leucyl-phenylalanine.

7.5.2 Removal of IgG4 antibodies partially restored peanut-induced mast cell activation

Plasma samples of twelve PS patients with IgE to the 3 major peanut allergens Ara h 1, Ara h 2 and Ara h 3 and with detectable peanut-specific IgG4 (Table 7.9), that had shown the ability to inhibit peanut-induced basophil and mast cell activation were selected, as well as nine plasma

samples from PA patients submitted to POIT (Table 7.8). Selected samples were depleted of IgG4 or mock-depleted, as confirmed by ELISA (Figure 7.3) and ImmunoCAP (Table 7.10). The inhibitory capacity of IgG4-depleted and mock-depleted samples was tested in the inhibition of mast cell activation assay using cells sensitised with plasma from PA patients who had the same IgE sensitisation pattern as PS samples, namely Ara h 1, Ara h 2 and Ara h 3 (Table 7.9).

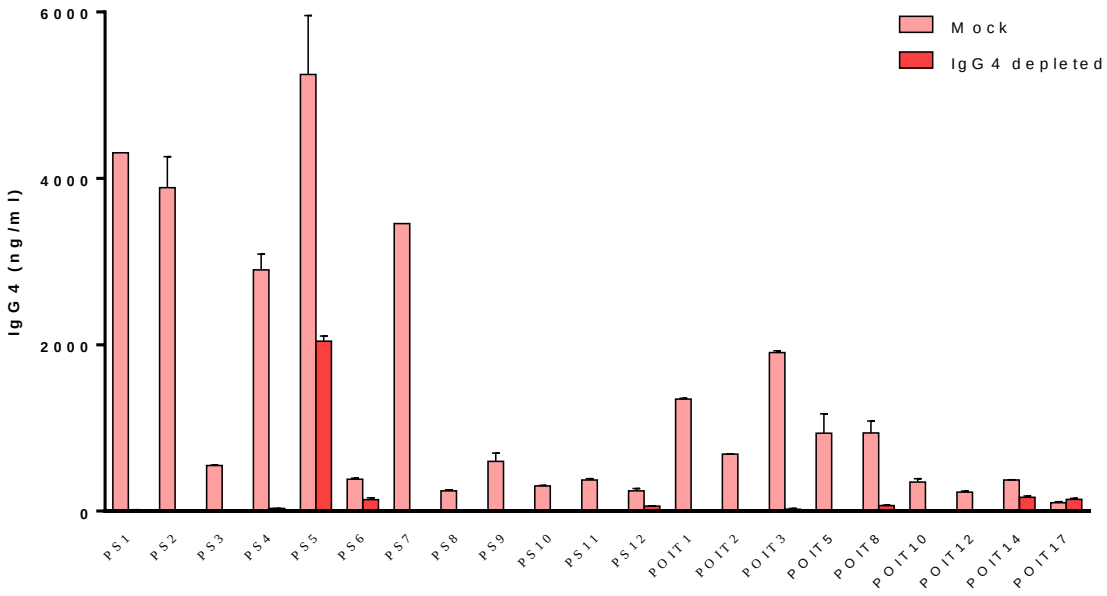

Figure 7.13 IgG4 ELISA of IgG4 and mock-depleted plasma samples.

Total IgG4 levels of plasma samples of 12 peanut-sensitised but tolerant patients (PS) and of 9 patients submitted to peanut oral immunotherapy (POIT), as assessed by ELISA.

Table 7.10 Peanut-specific IgG4 ImmunoCAP of IgG4 and mock-depleted plasma samples.

Levels of specific IgG4 (µg/ml) to peanut in plasma samples from peanut-sensitised but tolerant patients (PS, n=12) and patients submitted to peanut oral immunotherapy (POIT, n=9) following depletion of IgG4, as assessed by ImmunoCAP. Abbreviation: nd, not detectable

	Mock-depleted	IgG4-depleted
PS1	0.24	nd
PS2	0.62	nd
PS3	0.25	nd
PS4	0.49	nd
PS5	0.19	0.03
PS6	0.01	nd
PS7	0.10	nd
PS8	nd	nd
PS9	0.02	nd
PS10	0.46	nd
PS11	0.28	nd
PS12	0.11	nd
POIT1	1.99	nd
POIT2	2.29	nd
POIT3	1.10	nd
POIT5	0.27	nd
POIT8	2.22	nd
POIT10	2.31	nd
POIT12	0.29	nd
POIT14	1.58	0.05
POIT17	nd	nd

As the method adopted for IgG4 depletion required a 1 in 10 dilution of the plasma sample, pilot experiments were performed in which plasma samples of interest were tested in the mast cell inhibition assay diluted 1 in 10 to confirm that the diluted samples could still inhibit peanut-induced mast cell activation (Figure 7.14).

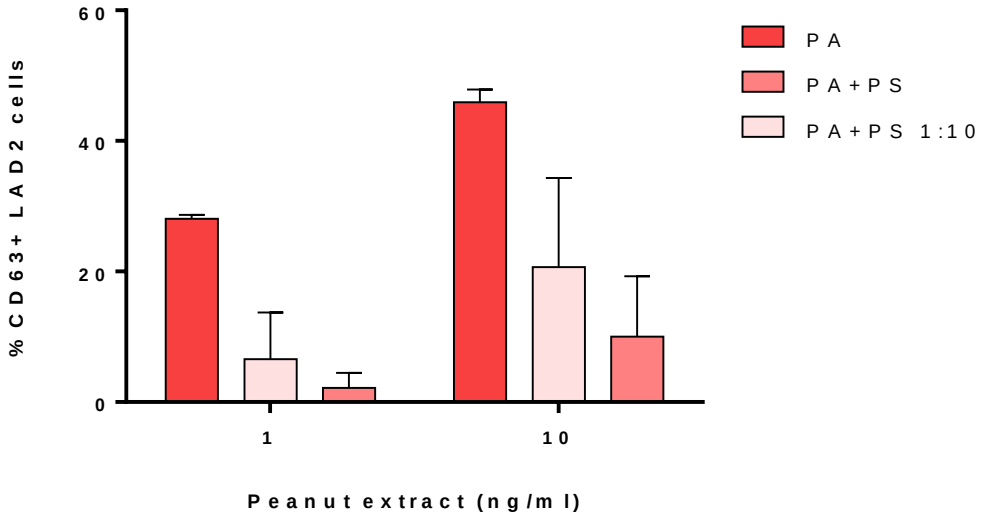

Figure 7.14 Peanut-induced activation of mast cells sensitised with plasma from a reference peanut allergic patient in the presence of plasma of peanut-sensitised but tolerant patients diluted 1:10.

PA, Peanut-induced activation of mast cells sensitised with a reference peanut allergic plasma (red); PA+PS, Peanut-induced activation of mast cells sensitised with a reference peanut allergic plasma in the presence of plasma from peanut-sensitised but tolerant patients, undiluted (dark pink) and diluted 1:10 (light pink).

IgG4 and mock-depleted samples were then re-tested in the mast cell inhibition assay using LAD2 cells sensitised with the reference plasma from a peanut allergic patient. IgG4 depletion from PS samples partially restored peanut-induced mast cell activation (p=0.007). Figure 7.15 represents the percentage of mast cell inhibition for IgG4- and mock-depleted samples of PS patients.

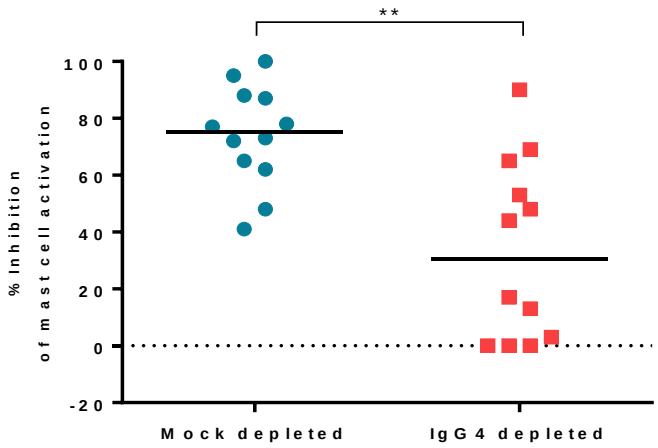

Figure 7.15 Inhibition of peanut-induced activation of mast cells sensitised with plasma from a peanut allergic patient in the presence of mock-depleted plasma samples or IgG4-depleted plasma samples from peanut-sensitised but tolerant patients

Median inhibition=75% versus 30%, respectively; p=0.007, n=12. %Inhibition = (%CD63+ of cells sensitised with PA plasma-%CD63+ of cells sensitised with PA plasma in presence of test plasma)/%CD63+ of cells sensitised with PA plasma.Horizontal lines represent the median for each group of patients. **p<0.01.

Reduction of mast cell inhibition was also observed when testing IgG4-depleted post-POIT samples. (p=0.040, Figure 7.16).

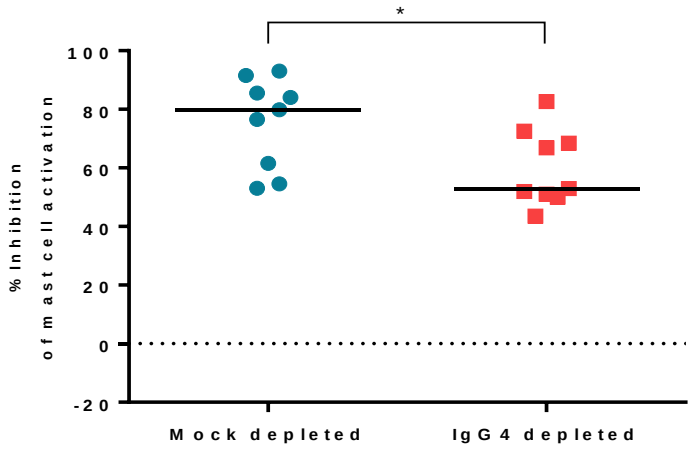

Figure 7.16 Inhibition of peanut-induced activation of mast cells sensitised with plasma from a PA patient in the presence of mock-depleted plasma samples or IgG4-depleted plasma samples from patients who underwent peanut oral immunotherapy

Median inhibition=80% versus 52% respectively; p=0.04, n=9. %Inhibition = (%CD63+ of cells sensitised with PA plasma-%CD63+ of cells sensitised with PA plasma in presence of test plasma)/%CD63+ of cells sensitised with PA plasma. Horizontal lines represent the median for each group of patients. *p<0.05.

7.6 Conclusions

At the population level, peanut allergic patients have higher levels of peanut-specific IgE to peanut and are more likely to have IgE directed to Ara h 2 and to the other peanut major allergens. At the individual level, peanut allergic and peanut tolerant patients can have comparable levels of specific IgE to peanut. Inhibition of IgE activity by allergen-specific IgG4 is a mechanism of peanut tolerance in patients with elevated specific IgE to peanut and its major peanut allergens. The clinical phenotype of individual peanut-sensitised patients is probably a result of the combination of different characteristics of the patient's pool of peanut-specific IgE antibodies, as well as of the inhibitory activity of peanut-specific IgG4 and potentially other immunoglobulin isotypes.

Chapter 8 Discussion

During my PhD, I have developed and validated a BAT to peanut as a novel biomarker of peanut allergy and as a biomarker to estimate the severity and threshold of allergic reactions to peanut. The BAT showed to be far superior in diagnosing peanut allergy than tests that detect the presence of peanut-specific IgE. As an *in vitro* surrogate of oral food challenge, BAT estimated the severity and the threshold of allergic reactions to peanut. The gating strategy used to identify basophils had a critical role in the enhanced performance of the basophil activation test. I have also used this assay in independent projects that detected the biological activity of peanut allergens in household dust samples and assessed the ability of auto-IgE antibodies present in the serum of asthmatic patients to degranulate mast cells and basophils. As a result of my MRC Early Career Award, having validated the BAT, I developed a novel mast cell assay using the LAD2 cell line to test the ability of samples from allergic and tolerant patients to elicit mast cell activation and inhibition, respectively. Finally, I have used these basophil and mast cell assays to explore the mechanisms underlying the discrepancy between the presence of IgE and clinical reactivity to peanut. I demonstrated that in some patients the absence of clinical allergy despite the presence of IgE is explained by peanut-specific IgE directed against irrelevant minor allergens. In other patients, with IgE directed to major peanut allergens, IgG4 can block the function of IgE preventing activation and degranulation of effector cells, mast cells and basophils.

8.1 The strategy adopted to gate on basophils has implications for the diagnostic accuracy of the basophil activation test

The BAT can be used to diagnose allergic disease and to study its underlying immunological mechanisms. The methodology used to identify basophils has important consequences for the outcome of the test. In chapter 3, I showed that CD123 is down-regulated following basophil activation and hence gating strategies that depend on this marker lead to the loss-to-analysis of activated basophils and to the underestimation of basophil activation. Using CD203c in addition to CD123/HLA-DR or CD203c in isolation proved superior to gating on CD123/HLA-DR, reducing the number of false-negatives and false-positives and improving the diagnostic accuracy from 91

to 97%. These results show that when BAT is used as an allergy test, the gating strategy adopted has important diagnostic implications in the assessment of individual patients.

Basophils have been identified with CD123 and HLA-DR in previous studies, using flow cytometry and other techniques[189], and CD123 expression was reported to be stable with the atopic status of the patient and following basophil activation[66, 69, 187]. In my study, the MFI of CD123-FITC was comparable between atopic and non-atopic patients, but there was a decrease in the MFI of CD123-FITC following basophil activation. This is in contrast to previous studies[66, 69, 187] and is probably related to differences in the study population (e.g. adults versus children), in the disease models studied (e.g. patients with respiratory versus food allergies) and in the study design (e.g. basophils stimulated with allergen in vivo versus in vitro, which can have different kinetics). With respect to HLA-DR, basophils were HLA-DR-negative and were consistently distinct from HLA-DR-positive cells under all conditions. These findings are consistent with previous reports[66] and with recent studies that basophils do not seem to have a role in antigen-presentation in humans[190, 191].

The combined use of the three basophil identification markers, CD203c/CD123/HLA-DR, proved superior to using CD123/HLA-DR alone. The diagnostic performance of BAT to peanut between identifying basophils as CD123+/HLA-DR- cells or as CD203c+/HLA-DR- cells was compared. The latter gating strategy resulted in a greater area under the ROC curve (0.99 versus 0.96) and improved the diagnostic accuracy (97% versus 91 %) of BAT. Furthermore, considering the minimum number of basophils of 500 as an exclusion criterion, 15% of patients would be inevaluable using CD123+/HLADR-. The consequences of the adopted gating strategy are clinically relevant as the BAT is used to diagnose peanut allergy in individual patients. An example is illustrated in Figure 3.6, in which, with basophils gated as CD123+/HLA-DR-, BAT would be considered negative at the diagnostic concentrations of 10 and 100 ng/ml. In fact, BAT, in this case, was clearly positive when basophils were selected using the 3 markers' strategy. A false-negative would have serious consequences, as it could lead to liberalization of peanut consumption, risking potentially severe allergic reactions. The loss-to-analysis of cells and/or the underestimation of basophil activation have important implications for the final outcome of the test and thus for the diagnosis of individual patients. The same applies to other clinical applications of BAT, such as monitoring of treatment, and to mechanistic experiments. Using the final gating strategy (SSC^{low}/ CD203c+/HLA-DR-), the contamination with other immune cells was minimal;

therefore, there did not seem to be particular advantage in using a lineage negative antibody mixture to exclude other immune cells before gating on the CD203c+/HLA-DR- basophils.

Identifying basophils with CD203c alone led to comparable outcome in terms of numbers of basophils and basophil activation markers to using CD203c+/HLA-DR- and was also superior to using CD123+/HLA-DR-, as represented in Table 3.3. CD203c is a basophil specific marker in whole blood. Expression of CD203c is constitutive but is increased in patients with atopic eczema and food allergy [104, 192], as previously described in terms of histamine release[193] suggesting it is a marker of underlying basophil activation, possibly reflecting ongoing piecemeal degranulation. This enhances the separation of CD203c+ basophils from the other blood cells in a population of highly atopic children[165], like the one studied here. The majority of patients in our study had eczema and other food allergies in addition to suspected peanut allergy and this represents a population where CD203c would be constitutively expressed at a higher level compared to other children, allowing a clear differentiation between basophils and other cells. The conjugation to the bright fluorochrome phyco-erythrin may have also contributed to optimal identification of cells expressing CD203c. SSC^{low}/CD203c+ is an alternative basophil identification strategy that has the advantage of serving also as an activation marker, enabling BAT to be performed as a 2-colour (preferred) or even as a single colour BAT, which would make BAT easier and less expensive to perform.

Despite the number of studies evaluating BAT as a diagnostic or clinical tool, the clinical and research applications of BAT are still surrounded by some controversy with regards to the preferred gating strategy to identify basophils by flow cytometry. Herein I compared different strategies for identifying basophils using CD123 and their clinical implications and demonstrated the down-regulation of CD123 following basophil activation. The performance of BAT soon after blood collection and the consistent use of live cells for flow cytometry on the same day contributed to the validity of the results. However, my study population consisted mostly of highly atopic children enrolled in a study examining the use of BAT in the diagnosis of peanut allergy[165]. Therefore, although my results would apply to other paediatric populations of patients with suspected peanut allergy, a similar validation would need to be conducted for application to other populations, such as non-atopic or older patients being assessed for other conditions such as drug allergy. While assessing changes in basophil identification markers, I discovered that in a subset of patients, basophils down-regulate CD123, the low affinity subunit of the IL-3 receptor,

upon activation. This phenomenon was patient-specific rather than specific for atopic or allergic status. In the patients where this was observed, the down-regulation occurred only in conditions where basophils were activated and correlated with the degree of activation, as expressed by CD63 and CD203c. IL-3 is predominantly produced by T cells and is able to induce basophils to release histamine and up-regulate CD203c and CD63 in the absence of allergen[194, 195]. It can also act synergistically with allergen or other stimulants to increase basophil activation and histamine release[70, 167, 194]. The response to this priming effect is variable between basophil donors and requires different concentrations of IL-3[186]. Some research groups have used exogenous IL-3 to prime basophils in the BAT[70, 167, 196]. However, basophils secrete IL-3 themselves in response to IgE-mediated activation for autocrine priming, which has been suggested to be a possible mechanism underlying the hyper-reactive nature of the basophils of allergic patients[197]. The basophil intracellular pathways down-stream of the IL-3 and the IgE receptors seem indeed connected[195]. Future studies should test the hypothesis that down-regulation of CD123, which is part of the IL-3 receptor, is a negative regulatory mechanism to avoid further cell activation.

8.2 The basophil activation test improved the diagnosis of peanut allergy

In order to arrive at a correct diagnosis of peanut allergy or tolerance, a considerable proportion of peanut-sensitised patients seen in Allergy clinics need to undergo an oral food challenge. Specialised centres have become over-whelmed with increasing numbers of oral food challenge requests; and over-diagnosis of peanut allergy due to over-reliance on allergy tests alone is common. Diagnostic cut-off levels have been determined for peanut allergy in some centres[127-131]. However, there is a large immunological grey area between 95% PPV and 95% NPV cut-offs for SPT, peanut-specific IgE and Ara h 2-specific IgE (Figure 1.6). If one applies a single cut-off value based on the ROC curve point-of-inflexion, the diagnostic accuracy of these tests suffers. In BAT, the ROC curve optimal cut-off acted simultaneously as positive and negative cut-off with no "immunological grey area", allowing for a significant reduction in the number of oral food challenges, even amongst difficult patients with conflicting history and results of SPT and specific IgE to peanut and peanut components. Unlike for these other tests, BAT allowed the use of the ROC point-of-inflexion as a single cut-off value while maintaining a 97% diagnostic accuracy.

This is the first study to prospectively validate BAT in an independent population and to evaluate its diagnostic performance on its own, in combination and sequentially with other allergy tests as well as its impact on the number of oral food challenges[74, 143, 198]. I studied a large population, including not only sensitised but also non-sensitised non-allergic patients. Although peanut-induced basophil activation would not be expected in the absence of peanut-specific IgE, it was important to demonstrate the specificity of BAT in NA patients. BAT maintained a good performance in an independent population, prospectively recruited to validate the diagnostic cut-offs. In 44 children with evidence of sensitisation and equivocal allergy test results, three specialist doctors were in poor agreement regarding diagnosis and were unable to decide in the majority of cases whether or not they were peanut allergic without doing an oral food challenge. In contrast, BAT performed very well with 95% diagnostic accuracy in this particularly challenging subgroup.

One of the strengths of the diagnostic study is that participants were carefully clinically phenotyped, the vast majority by oral food challenge. In the primary study population, twenty-three patients were assumed to have peanut allergy, based on SPT and/or peanut-specific IgE≥95%PPV cut-offs (previously validated in our clinic's patient population[129]) and recent clear-cut allergic reactions to peanut reported in the clinical history. This assumption represents a potential weakness of the study; however, given the extremely high probability that such patients would react clinically, it was decided on clinical and ethical grounds not to challenge them. The majority of patients that were challenged underwent DBPCFC (48/61), but 4 children younger than 1 year of age and another 9 older children underwent open oral food challenges. The majority (7/9) of older children undergoing open oral food challenges had negative challenges (open oral food challenge is the gold-standard for peanut tolerance) and the two who had a positive oral food challenge had objective unequivocal signs of an allergic reaction immediately after peanut ingestion, consistent with the new Practall guidelines' criteria for a positive oral food challenge[119]. In 5 patients (4.6%), the oral food challenges were inconclusive, which highlights the fact that, although DBPCFC is the gold-standard, it is not fool-proof in the diagnosis of peanut allergy[120]. BAT may prove particularly useful in cases where an oral food challenge cannot be performed or is indeterminate. In the external validation population, 94% of patients were challenged and all positive oral food challenges were DBPCFC.

It should be noted that a single day challenge protocol was used, in which placebo and active doses were interspersed (Table 2.1, Section 2.2.3.1.). This is not in keeping with the recent Practall guidelines[119] but is in keeping with the LEAP study recommended challenge procedure to peanut (registered at http://ClinicalTrials.gov with the identification number NCT00329784)[8, 199]. This is done for pragmatic reasons as a large number of children travel from further afield in the United Kingdom and it is difficult for them to stay for a 2-day oral food challenge. On the rare occasion that a child reacts following a placebo dose then a 2-day DBPCFC is performed in which placebo doses are administered on one day and active doses on the other day. Only 2 out of 52 patients with positive challenges in the severity and threshold study initially reacted following placebo on a mixed active-placebo one-day protocol and both these patients had their diagnosis of peanut allergy subsequently confirmed using a 2-day DBPCFC.

The main limitation of the use of BAT for diagnosis was that a proportion of patients had non-responsive basophils, rendering BAT uninterpretable. The proportion of non-responders found (11.5% in the primary study population and 6.2% in the external validation population) was similar to that previously described[104, 143, 200, 201]. This is analogous to situations where SPT cannot be interpreted because of a negative histamine control or where peanut-specific IgE cannot be interpreted in the light of a high polyclonal IgE production or indeed where an oral food challenge is inconclusive. Importantly, these are not misdiagnosed patients but cases where BAT is uninterpretable and the diagnostic work-up needs to be taken further, namely by doing an oral food challenge. The fact that non-responders were almost exclusively (92%) peanut-tolerant patients raises the question of whether basophil unresponsiveness through the IgE-mediated pathway could be a mechanism underlying peanut tolerance.

An important consideration is the composition of allergen extracts. In this study, different peanut extracts were used for different tests and could have accounted for differences in the results of SPT, specific IgE and the BAT and in their ability to diagnose peanut allergy. Nevertheless, the same ALK-Abelló peanut extract from the same batch was used for all the basophil and mast cell *in vitro* assays; the same manufacturer was used for the SPT performed in all patients; and the specific IgE assays from Thermofisher are well-standardised. Furthermore, all extracts contained the peanut major allergens (see section 2.3.3). The fact that the same manufacturer was used for SPT and BAT may have contributed to the observed closer performance between these two tests compared to the serologic tests.

There are a number of other considerations when considering applying BAT in clinical practice. BAT needs to be performed on live cells, soon after blood collection, and requires expensive flow cytometry equipment and appropriately trained staff. Appropriate standardisation of the laboratory procedure, flow cytometry and data analysis would be required for a wider application of the test. The development of automated methods and the use of simplified equipment are desirable for routine clinical application of the BAT.

Following the evaluation of the diagnostic performance of the BAT and the other allergy tests by ROC curve analysis, the utility of the different allergy tests in the diagnosis of peanut allergy in clinical practice, and their impact on the reduction of oral food challenges, comparing this with the current strategy adopted in our clinic of combining SPT and specific IgE to peanut was assessed. Very few studies have addressed the utility of combinations of allergy tests and this deficiency has been highlighted as an unmet clinical need in the NIAID-sponsored food allergy guidelines[3]. This was assessed in three ways: considering each test on its own, considering the results of different diagnostic tests simultaneously, and considering BAT as a second or third sequential step in the diagnostic process, performed in patients where the results of single or combinations of standard allergy tests were equivocal. For these analyses, the entire primary study population was considered, including patients with non-responder basophils, and interpreted the results of the different tests by applying conventional diagnostic cut-offs[44, 129]. The results of tests when interpreted individually were considered equivocal if they fell between the negative and the positive conventional cut-offs for SPT, peanut-specific IgE[129] and Ara h 2-specific IgE[44] or when different allergy tests gave contradictory results. The change in the number of oral food challenges was compared with the strategy of combining SPT and specific IgE currently adopted in our clinic to decide about the need for oral food challenge.

The impact of BAT was different in the three scenarios considered: single tests, combination of tests, and BAT as a sequential step in the diagnostic process (Table 4.10). Considering single tests, BAT performed best, followed closely by Ara h2-specific IgE and SPT, even when patients with non-responder basophils were taken into account. Peanut-specific IgE performed the poorest and required the highest number of oral food challenges. BAT is a unique test as it is a functional test and does not present any "immunological grey area" for the patients for whom there is an outcome. The same cut-off (based on the ROC point of inflexion 4.78%) that allowed confirmation of peanut allergy with 95% certainty also allowed the exclusion of the diagnosis with 98%

certainty. The positive likelihood ratio (LR+) of the elected cut-offs of BAT was 24.4 for the point of inflexion which is above the level of 10 at which a test is clinically useful[133], resulting in a high post-test probability irrespective of the pre-test probability. Conversely, the negative likelihood ratio of BAT was extremely low, which proves very useful in excluding the diagnosis of peanut allergy given a negative test result.

Surprisingly, the use of different combinations of tests provided little, if any, advantage compared to BAT alone, with a uniform reduction in the percent of correct diagnoses and a significant increase in the number of oral food challenges required. Disappointingly, the combination of tests did not result in a consistent decrease in the number of false-negative outcomes. When combining results of different diagnostic tests before deciding about the need for oral food challenge, the best outcome was to combine two tests among SPT, Ara h 2-specific IgE and BAT. Adding more tests (i.e. considering the results of 3 or 4 tests) did not significantly reduce and often increased the number of oral food challenge because in the cases where the tests gave contradictory results, oral food challenges were required. Combining two allergy tests compared to BAT alone decreased the false-negative cases by 2% (SPT + BAT) or decreased the false-positive cases by 1% (Ara h 2-specific IgE + BAT), depending on the specific combination. In particular, adding Ara h 2-specific IgE to SPT and specific IgE to peanut did not improve the diagnosis. Adding specific IgE to peanut to other tests resulted in no change or in an increase in the number of oral food challenge, further putting into question the diagnostic value of specific IgE to peanut when other tests are available.

Performing BAT as a sequential step reduced the number of BAT tests required (Table 4.10, Figure 4.7) and had a major impact in reducing the number of oral food challenges regardless of the test performed as first line. For instance, performing BAT following SPT or following Ara h 2-specific IgE allowed a 97% reduction in the number of oral food challenges compared to the combination of SPT and peanut-specific IgE (the routine clinical reference-point in our clinic) and a 92% reduction compared to BAT alone. However, this was at the expense of 2 or 3 false-negative outcomes. To prevent any false-negative cases from occurring as a result of this sequential test approach, all the BAT-negative patients would require oral food challenges in addition to the patients with equivocal BAT. Even in this more conservative scenario the total number of oral food challenges required would be significantly reduced by 64% (SPT followed by BAT) or 69% (Ara h 2-specific IgE followed by BAT) compared to combining SPT and peanut-

specific IgE. The decision on whether to increase the number of oral food challenges or of BAT, both reducing the possibility of false-negative tests, would depend on a cost-benefit analysis. I believe that SPT followed by BAT is better than Ara h 2-specific IgE followed by BAT for practical reasons (SPT provides immediate results while Ara h 2-specific IgE followed by BAT would require 2 separate blood collections) and given regional differences in the patterns of sensitisation to peanut allergens[39]. The 3-step diagnostic strategy further reduced the number of BAT required and eliminated the need for oral food challenges but this was at the expense of a higher false-negative rate, not from BAT but from SPT and Ara h 2-specific IgE.

A sequential approach was previously proposed by Dang et al[44] to test the accuracy and practical applicability of Ara h 2-specific IgE in the diagnosis of peanut allergy. The exact same approach of considering Ara h 2-specific IgE as a second step in the diagnosis of peanut allergy in cases where SPT or specific IgE to peanut were equivocal was applied to our own study population. BAT correctly diagnosed a higher proportion of patients, resulted in a lower number of false-negative diagnosis and a greater reduction in the number of oral food challenges, compared to Ara h 2-specific IgE. One possible explanation for the superiority of BAT compared to Ara h 2-specific IgE as a diagnostic test is that BAT is a functional assay that takes into account the combined ability of different IgE molecules, when cross-linked by different peanut allergens present in the extract, to trigger basophil degranulation. This includes both high and low affinity IgE antibodies directed to major and minor peanut allergens (not only Ara h 2 and some of which were not tested on ImmunoCAP). Besides, Ara h 2 is not always the most dominant peanut allergen in all geographical locations[39]. Even in areas where Ara h 2 is dominant, such as the United States, Australia and the United Kingdom, studies have reported cases of peanut allergy in which patients test negative to all available peanut components[43, 44, 135]. Also in this study, there was one case of a peanut allergic child with detectable IgE to peanut extract but undetectable specific IgE to Ara h 2 and to the other peanut components.

8.3 Estimating the severity and the threshold of allergic reactions to peanut with the basophil activation test

Current management of peanut allergy relies on allergen avoidance and the prescription of auto-injectable adrenaline to patients deemed to be at risk of anaphylaxis. Knowing whether individual

patients are at risk of reacting to low amounts of the allergen or of developing severe reactions would improve the care for patients with peanut allergy. Apart from resembling very closely the clinical phenotype of patients in terms of clinical reactivity to peanut[165], BAT reflected the severity and the threshold of allergic reactions to peanut. Allergen-specific basophil reactivity (as measured by CD63 peanut/anti-IgE) and basophil sensitivity (as measured by CD_{sens}) were identified as biomarkers of severity and threshold of allergic reactions to peanut during oral food challenge.

This was a prospective study of a well-characterised population of peanut allergic patients that were submitted to oral food challenge regardless of the presence of clinical risk factors for severe reactions and of the SPT and specific IgE results. In most previously published studies, patients with a history of anaphylaxis, current asthma and/or with specific IgE levels above the 95% positive predictive value cut-off were often excluded, thereby limiting the spectrum of the disease severity studied[202]. Different severity scores have been adopted in different studies, some including both symptom score and the eliciting dose. The severity score adopted here had previously been validated[148, 203] and does not include the dose that caused a reaction as the aim was to assess these two factors, severity and threshold, independently. Indeed, distinct BAT parameters reflected the severity and the threshold of allergic reactions. The best parameter to predict severity was the ratio between basophil specific activation in response to allergen and basophil non-specific activation in response to anti-IgE. The response of basophils of allergic patients to allergen has been reported to be greater than to anti-IgE or anti-FcεRI[192, 200]. In a previous study of cow's milk allergic children, the ratio between the percentage of CD63-positive basophils in response to cow's milk and to anti-FcεRI was higher in patients with persistent cow's milk allergy compared with patients who outgrew their allergy and was correlated with the severity of the reaction during challenges[200].

In contrast to a study recently published[204], in which BAT showed no correlation with severity but only with threshold, in the present study, BAT informed not only about threshold but also about severity of allergic reactions during oral food challenge. Although different severity scores were primarily used, in both studies findings were confirmed with other severity scores; thus the severity scores used were unlikely to have accounted for the discrepancy between study results. These differences might be explained by the adopted oral food challenge protocol. In the cited study, oral food challenges were performed over 2 days, with 2-hour intervals between doses up

to a cumulative dose of 4.443g of peanut protein[204]. In the present study, oral food challenges were performed on a single day with 20-minute intervals between doses and the cumulative dose for children older than 3 years was 9.35g[205]. These factors could have contributed to the greater severity of reactions during the oral food challenge observed, enabling to identify a biomarker for severity. Stronger correlations for threshold were found, logistic regression analyses were performed and cut-offs both for severity and threshold were determined in this study[205]. Limitations related to the design and performance of the challenges need to be taken into consideration, namely the fact that the lowest dose of peanut protein given was 0.033g for high-risk patients and 0.1g for most patients which is above the published estimated doses calculated to cause objective symptoms in 10% of the peanut allergic population (ca. 3mg peanut protein); and the fact that challenges were performed with interspersed doses which resulted in variable time interval between active doses in different patients. With regards to the assessment of the severity of allergic reactions, there may have been some variation in symptom scoring by different researchers undertaking the challenges. A list of subjective symptoms and objective signs to be ticked by the operator would have been preferable compared to free text for the description of the allergic reactions that was used in the case report form.

SPT and serum specific IgE to peanut have been tested as biomarkers of the severity of allergic reactions to peanut. Previous studies showed contradictory results, some finding that SPT and serum specific IgE predicted the development of anaphylaxis[26, 206-208] and others failing to find any association between the allergy test results and severity of allergic reactions[24, 202]. In my study, patients with severe reactions had higher levels of specific IgE to peanut, to Ara h 1 and to Ara h 2. Severity has also been associated with recognition of a greater number of peanut allergens[209, 210] and epitopes[211] by patients' IgE and with increased intensity of bands on immunoblotting as a surrogate for antibody affinity and avidity[209]. My study corroborates these findings as patients with severe reactions had IgE directed to a larger number of peanut major allergens compared with patients with mild/moderate reactions. Interestingly, patients with a higher ratio of peanut-specific IgG4 to IgE reacted at higher doses of peanut, supporting the hypothesis that IgG4 competes with IgE for binding to the allergen, blocking its effect and preventing degranulation of basophils at low concentrations of peanut extract. One of the advantages of BAT is that it is a functional assay that takes into account all these factors, including levels, specificity, diversity and affinity of allergen-specific IgE and even possible interference by other allergen-specific antibodies, which together

are responsible for allergen-induced effector cell activation. Therefore, BAT has a greater potential to reflect the allergic reaction as it happens *in vivo* than methods that test these IgE parameters separately. This is reflected in my data where, following multivariable analyses, BAT parameters proved to be more predictive of severity and threshold of allergic reactions than the other tests.

A relationship between severity and threshold has previously been suggested[212] with patients who react to lower doses being more at risk of developing severe symptoms. In my study and others[204, 213], the clinical parameters of severity and threshold were not correlated. However, a strong correlation was found for the respective basophil parameters. This discrepancy could be due to the fact that during the BAT the "*in vitro* challenge" can proceed to higher doses whereas *in vivo* oral food challenges are typically stopped with the first allergic symptoms and signs. The severity of allergic reactions during oral food challenges may have been different if a large dose of allergen had been consumed at once. The fact that basophil activation can be tested at high doses of allergen, regardless of disease severity, is another clear advantage of BAT as a biomarker of disease severity compared to oral food challenges.

The BAT markers identified for severity and threshold of reactions during oral food challenge with peanut may not reflect the severity and threshold of allergic reactions in the wider community. Hourihane et al[208] showed that the challenge score correlated with the most recent reaction but not with the most severe reaction in the community, suggesting that patients' reactivity to peanut changes over time. Co-factors that can increase the severity of allergic reactions (e.g. uncontrolled asthma, viral infections, menstruation, exercise, consumption of alcohol or drugs and psychological factors) may be present in the community and are usually controlled during oral food challenge. Similarly, variation in the threshold may occur as the eliciting doses during oral food challenge can be underestimated, e.g. because the patient feels safe during the oral food challenge, but can also be overestimated, e.g. because mild symptoms would remain unnoticed in a community setting[175]. Severity and eliciting dose during challenges have not been reproducible in other studies, highlighting the limitations of this approach[214, 215].

The utility of BAT as a marker for severity and threshold of allergic reactions is to provide additional information to the patients. This information should be interpreted in the context of the clinical history and the presence of other risk factors. The management of patients should continue to be based on patient education and the importance of an emergency treatment plan

and appropriate training cannot be over-emphasised, regardless of the magnitude of the allergy test results, including BAT. However, BAT can identify patients who are at risk of reacting to small amounts of the allergens and of developing severe symptoms who require special attention. Further validation of these objective BAT markers in different populations repeated at different time points may allow us to identify the subset of high-risk peanut allergic children who may require closer monitoring as well as the subgroup of children whose allergy to peanut may be spontaneously resolving. Identification of high-risk groups should not be based only on the biological markers but also on psycho-social, demographic, behavioural and clinical parameters.

8.4 Additional applications of the basophil activation test

Being an *in vitro* surrogate for IgE-mediated reactions, the basophil activation test has an immense potential apart from its use for the diagnosis of allergic disease.

8.4.1 Determination of biological activity of allergens using the basophil activation test

Previous observational evidence demonstrated that sensitisation to peanut may occur through the skin[88]. Household peanut consumption, which is a marker for environmental peanut exposure, was identified as a risk factor for the development of peanut allergy[145] and was positively correlated with peanut protein levels in the home environment[213]. Following on from these findings, I determined that peanut protein in house dust was biologically active and able to activate basophils from peanut allergic children[213].

The presence of biologically active peanut protein detectable in dust supports the concept that environmental peanut exposure can lead to peanut allergic sensitisation. To give more weight to the notion that peanut in dust can sensitise young children, the aim was to confirm that the peanut levels that were measured in house dust were biologically significant in peanut allergic patients. The ability of peanut protein in dust to cause allergen-specific activation of basophils from children with peanut allergy was indeed demonstrated. Peanut protein in household dust induced a dose-dependent activation of basophils from children with peanut allergy, resulting in a bell-shaped dose-response curve typical of allergen-induced basophil activation, comparable with the dose-response to similar concentrations of an independent peanut standard. These observations are

highly suggestive of a peanut-specific basophil response. Given that these children were solely allergic to peanut and neither sensitised nor allergic to any other food or airborne allergen, basophil activation was very unlikely to have been caused by allergens other than peanut present in the dust samples (e.g., house dust mite or grass and tree pollen allergens). The allergen-specific response was further confirmed by the fact that activation was not observed when basophils from non-allergic children were stimulated with the high peanut level–containing dust samples. Furthermore, the inability of dust samples with negligible levels of peanut to cause basophil activation in both children with peanut allergy and non-allergic children excludes non–allergen-specific basophil activation. Taken together, these findings confirm that peanut allergens in house dust are able to interact with immune cells, such as basophils. I supposed that, being biologically active, peanut allergens in house dust are not only able to activate mast cells and basophils but also to be captured by dendritic cells and presented to T cells in the context of a Th2-driven immune response leading to allergic sensitisation.

8.4.2 *In vitro* basophil and mast cell assays as surrogates for *in vivo* tests for autoimmunity

In a collaborative project, using a novel quantitative calibrated ELISA method, a subset of asthmatics were identified as having circulating anti-IgE antibodies in higher levels compared with non-atopic non-asthmatic controls, irrespective of their atopic status. These anti-IgE antibodies were not present in all asthmatics studied and did not distinguish atopic and non-atopic asthmatics. Furthermore, their levels were not always associated with their function. Some of these anti-IgE antibodies were able to elicit basophil activation whilst others interfered with the allergen-IgE interaction resulting in inhibition of allergen-induced basophil activation.

Activatory anti-IgE auto-antibodies could be responsible for asthma exacerbations in patients with intrinsic asthma or in patients with allergic asthma when asthma exacerbations develop independently of allergen exposure. Inhibitory anti-IgE auto-antibodies could explain the poor correlation between their levels and their function in some patients[216, 217]. It could also explain why some patients with allergen-specific IgE do not respond clinically when exposed to allergens and why some asthmatics do not improve when treated with omalizumab. The vigorous IgG4 response to allergen-specific immunotherapy could potentially include anti-IgE antibodies. The role of basophil-activating anti-IgE has been associated with the failure of immunotherapy

treatment[218]; however, inhibitory auto-antibodies could also be implicated. Similarly, inhibitory anti-IgE antibodies could be involved in the natural development of food tolerance and in resolution of existing food allergies, but this requires further studies.

Of note, the activities of these auto-anti-IgEs are quite different from those of omalizumab, the monoclonal antibody anti-IgE used therapeutically. Firstly, they seem to bind to different portions of the IgE molecule. Previous studies suggest that the majority of anti-IgE auto-antibodies recognize the Cε2 or Cε4 domains of IgE, and not the Cε3 domain to which omalizumab binds and which is thought to be inaccessible when the IgE is bound to its high- or low-affinity receptors. Secondly, they have different mechanisms of action. Omalizumab exerts its clinical effects by preventing binding of IgE to its high- and low-affinity receptors, and does not appear to reduce the intrinsic sensitivity of basophils to activation. On the contrary, recent experiments by MacGlashan and colleagues suggest that sequestration of IgE during omalizumab therapy renders basophils hypersensitive to activation by cross-linking of reduced surface-bound IgE molecules, at least partly by resetting of FcεRI coupled intracellular signalling[219, 220]. The auto-anti-IgE antibodies studied here were either activatory or inhibitory. The former induced basophil and mast cell degranulation by cross-linking of receptor-bound IgEs, similar to what happens following exposure to allergen. The latter seemed to exert their effect by interfering with the allergen-IgE interaction, i.e. by preventing binding of allergen to IgE they prevent mast cell and basophil activation.

8.4.3 Basophil intracellular signalling

It is possible to assess the phosphorylation of intracellular signalling mediators at the same time as assessing the up-regulation of activation markers on the surface of basophils by flow cytometry. It is important to ensure that, despite permeabilisation of the cell membrane, CD63 which is present on the membrane of intracellular granules is not detected on the surface of resting cells. Although this marker is present on the surface of granules inside the cell, it only becomes expressed on the plasma membrane at the cell surface after basophil activation. Flow cytometry allows the study of intracellular signalling in a heterogeneous mixture of blood cells, as opposed to western blotting which requires cell purification. Using flow cytometry, I assessed the phosphorylation of activatory intracellular signalling molecules down-stream of FcεRI, such as Syk and p38MAPK, and simultaneously assessed changes in the expression of activation markers in basophils, such as CD63. Studying cell signalling is valuable in addressing the mechanisms of

effector cell activation and inhibition. Ideally, I would also have liked to assess the changes in phosphorylation of the inhibitory mediators such as SHIP; however, there are no appropriate monoclonal antibodies available for use in flow cytometry.

8.5 Mechanisms of peanut allergy and peanut tolerance: differences in IgE or IgE inhibition?

Peanut-specific IgE does not equate to clinical peanut allergy. The majority of peanut-sensitised patients are peanut-tolerant, i.e. are able to eat age-appropriate amounts of peanut without developing an allergic reaction. It is possible that different mechanisms underpin tolerance to peanut in different subgroups of PS patients. In some cases, the absence of clinical reactivity in PS patients is due to the specificity of IgE for peanut proteins which are not able to cause degranulation of effector cells leading to systemic reactions; for example, PS patients with IgE that does not recognise any of the major peanut allergens and binds only to Ara h 8 or Ara h 9 in the cohort studied here. In other cases, particularly of PS patients with IgE directed to the major peanut allergens (such as Ara h 1, Ara h 2 and Ara h 3) that are known to be able to elicit effector cell activation and degranulation[46, 47], and would otherwise be a strong predictor of peanut allergy, inhibition of IgE by blocking antibodies may underlie the absence of allergic symptoms following peanut ingestion. In this study, I showed that plasma of PS patients were able to inhibit IgE-mediated peanut-induced basophil and mast cell activation, to a similar extent as plasma from patients submitted to POIT, and that this effect was partially mediated by IgG4. These findings suggest that oral tolerance to foods is in part mediated by IgG4 in a subset of patients that produce IgE antibodies and may also explain why there are so many more sensitised than allergic individuals.

Using passive sensitisation mast cell and basophil assays, previous findings in a whole blood basophil activation assay[165] were reproduced and it was confirmed that the factors responsible for allergen-induced effector cell activation and unresponsiveness in PA and PS patients are present in the plasma. Other evidence supports a role for plasma rather than cellular intrinsic factors in clinical reactivity to peanut. Clinically, PS patients who are able to eat peanut without any problems are often allergic to other foods and airborne allergens. This indicates that their basophils and mast cells are functional and able to respond to allergens. Experimentally,

basophils from PA and PS patients have similar IgE receptor expression on their surface and are able respond to IgE-mediated stimulants other than allergen[156, 221]. Therefore, intrinsic differences in cell reactivity between PA and PS children seem an unlikely explanation for the discrepancy between allergy and sensitisation.

The use of LAD2 cells in the passive sensitisation assays offered additional advantages compared to using primary human basophils. Firstly, being a cell line, LAD2 cells are readily available as they are stable in culture for long periods of time. Secondly, as they maintain their characteristics over time, LAD2 cells constitute a source of standardised effector cells, enabling the comparison of the effect of plasma from different patients, namely PA, PS, NA and patients undergoing peanut immunotherapy, in a reliable and consistent way. Finally, being mast cells, LAD2 cells allow the study of the other type of effector cells involved in the immediate phase of acute allergic reactions, apart from basophils. These mast cell assays may become particularly useful tools to explore the mechanisms of peanut allergy and tolerance (as they allow the study of the role of different serological components, namely of different antibody isotypes, in IgE-mediated peanut-induced mast cell reactivity) and also to assess the response to immunomodulatory treatment for peanut and other food allergies and allergic diseases as a biomarker for clinical improvement, in the future.

At the population level, the median serum levels of peanut-specific IgE were higher in PA compared to PS patients. This has been documented in different studies and has formed the basis of the development of diagnostic cut-offs for peanut-specific IgE[128, 129]. However, at the level of the individual, there was a substantial overlap between PA and PS patients. As described in section 4.5, a subgroup of peanut-sensitised patients with equivocal diagnosis showed no statistically significant differences in the levels of specific IgE to peanut components between PA and PS patients, except for Ara h 2[165]. PA patients were more likely to have IgE directed to Ara h 2 alone or to Ara h 2 in combination with the other peanut major allergens compared to PS subjects, and had higher levels on average. However, some PA patients did not have detectable IgE to Ara h 2 and conversely some PS patients had Ara h 2-specific IgE above the cut-offs that have been identified as being associated with a high probability of clinical peanut allergy[44, 165]. Various examples of PA and PS patients with the same IgE sensitisation pattern to peanut allergens but with opposite clinical outcomes could be found in this cohort and are shown in Tables 7.4 and 7.9. Taken together these observations demonstrate that the levels and specificity

of peanut-specific IgE do not account for the differences in allergic reactivity to peanut in all cases of PA and PS patients.

The hypothesis of a peanut-specific antibody counteracting the ability of peanut-specific IgE to activate and degranulate basophils and mast cells becomes particularly plausible in cases where peanut-specific IgE is high and/or when it is directed to peanut allergens that are known to be potent elicitors of effector cell activation, such as Ara h 1, Ara h 2 and Ara h 3. In these cases, IgG4 antibodies, and possibly peanut-specific antibodies of other isotypes, block IgE either by competing with IgE for binding to the peanut allergens or by inhibiting an activatory response at the cellular level by co-cross linking of IgE and ITIM-associated receptors, to which the inhibitory antibodies bind[222]. In my study, peanut-specific IgG4 levels were higher in PS compared to PA patients but the levels of IgG4 to peanut components were similar between the two groups, except for Ara h 2. Also in a previous study of egg allergic children, specific IgG4 levels to ovomucoid or ovalbumin were not significantly different between baked egg-allergic and tolerant children. My findings regarding IgG4 were similar in PS samples and samples of patients submitted to POIT. This is consistent with other POIT studies[110, 223, 224] where IgG4 has been reported to increase substantially with treatment and is thought to have a role as a blocking antibody. In some PA and NA patients with detectable peanut-specific IgG4 these antibodies are likely to be directed to non-allergenic components of peanut or to different epitopes of peanut allergens compared to IgE and thus have no ability to block the effect of IgE. In PA patients, this results in IgE-mediated effector cell reactivity whereas in NA patients tolerance results from the absence of peanut-specific IgE in the first instance. Furthermore, in NA subjects, the absence of inflammatory response against the allergens and the fact that B cells are not affinity matured are likely to result in IgG4 antibodies of low affinity that are not able to block IgE.

The relative amounts of IgG4 compared to IgE, rather than absolute antibody levels, is likely to be an important factor driving IgE inhibition. The ratio of IgG4 to IgE to peanut was significantly higher in PS compared to PA patients, indicating that the excess of peanut-specific IgG4 in relation to IgE could block peanut-specific IgE and contribute to the absence of clinical reactions to peanut in PS patients. In the literature, IgE/IgG4 ratios are usually reported and are often not corrected for the fact that the levels of IgE and IgG4 are usually measured in different units (IgE in KU_A/l and IgG4 in mg/l)[166, 225, 226]. I have calculated IgG4/IgE ratios considering the absolute amounts of these immunoglobulins in nanograms present in the serum of each patient at any

given moment which more accurately reflects the balance between the two types of antibodies that potentially have opposite effects. The ratio of IgG4/IgE to the peanut major allergens was also higher in PS, particularly to Ara h 2 which is believed to be the most potent elicitor of effector cell degranulation and consequently of allergic reactions to peanut[46]. My findings are consistent with previous observations in patients submitted to POIT where the increase in specific IgG4 was greatest for Ara h 2-specific IgG4 compared with IgG4 directed to the other peanut allergens[227] or where the increase in Ara h 2-specific IgG4 matched the sensitisation profile of existing IgE[114]. These observations support the concept that Ara h 2 is a dominant allergen in peanut allergy and that IgG4 responses associated with clinical improvement tend to counteract existing IgE responses.

The role of IgG4 in IgE inhibition in PS and POIT samples was confirmed by depleting this antibody subclass from patients' plasma and observing an overall increase in peanut-induced mast cell activation. The fact that IgG4-depletion had only a partial effect suggests that other antibody isotypes could also have an inhibitory effect on IgE. For example, peanut-specific IgA antibodies could contribute to the competition with IgE for binding to peanut allergens and contribute to the overall IgE inhibitory effect; however, in this population serum peanut-specific IgA was only detectable in a minority of patients and did not show any differences between PA and PS groups. This, however, does not exclude a possible role for secretory IgA at mucosal surfaces which is not present in significant levels in the plasma. Although in a mouse model of anaphylaxis, circulating IgA rather than enteric IgA were able to suppress IgE-mediated anaphylaxis[228], in a previous study of grass pollen immunotherapy, IgA isolated from the serum of treated patients did not show blocking activity[229]. Peanut-specific antibodies of other isotypes could also have some blocking effect but have not been measured in this study as there are currently no validated assays to quantify absolute levels of such allergen-specific antibodies.

There are other factors that may be explored in order to obtain a complete understanding of the mechanisms by which IgE and allergen may or may not be able to elicit effector cell activation that is responsible for the clinical manifestations of allergic disease. These include the role of IgE affinity as well as the specific epitopes to which IgE binds[230]. It is possible that two IgE molecules that are specific for the same peanut allergen recognize different epitopes in the allergen molecule or bind to the same epitope with different affinities leading to more or less potent effector cell activation. The determination of IgE and IgG4 binding to linear peanut peptides with

the aim to identify the dominant IgE and IgG4 epitopes on peanut allergens is an ongoing project described in Section 8.6.4. With regards to IgE affinity, preliminary experiments were performed using surface plasmon resonance to measure affinity of IgE for the purified peanut allergen Ara h 2 and compare it between plasma samples of PA and PS patients. However these experiments were not pursued due to the various technical limitations of this approach. Firstly, these experiments were restricted by the low concentration of allergen-specific IgE in plasma samples, of which there was limited volume; secondly, the patients' polyclonal IgE response had varying specific activity (i.e. proportion of IgE that is allergen-specific); and finally, non-specific binding of plasma proteins to the sensor chip surface prevented the detection of genuine allergen binding by surface plasmon resonance. IgG depletion from plasma prior to surface plasmon resonance and the use of different chemical methods to remove the proteins bound to the sensor chip did not resolve the latter problem. An alternative approach would be to purify IgE and run purified IgE over the cell containing the IgG4-Fc(sFcεRIα)$_2$ fusion protein; however, this would require large volume of samples, which were not available from the most interesting cases of peanut sensitised but tolerant infants and young children. Another alternative approach would be to use monoclonal antibodies generated from single peripheral blood B cells (described in section 8.6.4.). Monoclonal IgE antibodies bound to the IgG4-Fc(sFcεRIα)$_2$ fusion protein or captured by anti-IgE antibodies previously coupled to the sensor chip would be ideal to study the affinity of IgE for Ara h 2. This way the issues of low concentration of allergen-specific IgE, polyclonality of the IgE response, low volume of samples and unspecific binding to the cell surface would be obviated.

Christensen et al[231], using a panel of recombinant monoclonal anti-Der p 2 IgE antibodies with defined epitope specificity, clonality and affinity in different combinations, showed very elegantly that greater specific IgE concentration, greater specific activity to Der p 2 and greater epitope diversity increased basophil reactivity whereas greater affinity increased basophil sensitivity. Arguably, in individual peanut-sensitised patients, the clinical phenotype results from a combination of characteristics of the existing pool of peanut-specific IgE antibodies directed to a certain allergen, as well as of the inhibitory activity of IgG4 and possibly other immunoglobulin isotypes. Additionally, intrinsic differences in the IgG4 molecules relating to affinity and epitope specificity between PA and PS patients that could be responsible for inhibiting mast cell and basophil activation[109].

In summary, both differences in IgE and IgE inhibition can explain the discrepancy between allergic sensitisation and clinical allergy to peanut. In the population studied, patients submitted to immunotherapy fit the "desensitisation" profile described in Section 1.8.1.; whereas PS patients can correspond to the "stable sensitisation", "post-allergic sensitisation" or even the "antenatal sensitisation" or the "pre-allergic sensitisation" profiles. In any case, the clinical phenotype, of allergy or tolerance, is likely to result from a combination of relative amounts, specificity, affinity and clonality of IgE antibodies as well as of possible inhibitory antibodies such as IgG4, specific for the allergen.

8.6 Future perspectives

8.6.1 Application of the basophil activation test to participants in the LEAP and LEAP-On studies

The LEAP study is a randomised controlled trial aimed to assess whether early introduction of peanut prevents the development of peanut allergy[8], as described in section 2.1.2. The primary outcome is the prevalence of peanut allergy, determined by oral food challenge, at the age of 60 months (5 years). In a follow-on study (the LEAP-On study), participants in both arms of the LEAP study will avoid peanut for 1 year and will undergo another oral food challenge at the age of 72 months (6 years). The LEAP-On study addresses the question as to whether early introduction of peanut induces transient desensitisation or long-term tolerance raised by the fact that some infants were already sensitised when peanut was introduced in their diet at the start of the study. Participants in the LEAP and LEAP-On studies have been assessed at recruitment, at 12, 30, 60 and 72 months of age and blood samples have been collected at all these time points. Following the validation of BAT to peanut during my PhD, BAT has been performed in the LEAP and LEAP-On studies (2 time-points) under my direct supervision in real-time on the day when participants undergo the oral food challenge. This is supported by funding obtained from the Immune Tolerance Network and the National Institute of Allergy and Infectious Diseases, in the United States of America.

The performance of the basophil activation test using samples collected from participants in the LEAP and LEAP-On studies will further the scientific achievements reported in the present PhD

thesis. The data analyses at the end of the LEAP and at the end of the LEAP-On studies will allow accomplishing the following objectives:

1. Further validate the BAT as a diagnostic marker for peanut allergy as it correlates very closely with disease expression at the level of the individual;

2. In secondary analyses, BAT may be used as a surrogate marker of peanut allergy versus tolerance, i.e. as a secondary endpoint, in study participants where standard clinical methods have failed, namely in children who refused an oral food challenge or whose oral food challenge had an indeterminate outcome;

3. Validate the use of BAT to predict the severity and the threshold of allergic reactions to peanut during DBPCFC;

4. Assess the results of BAT and the other immunologic outcomes (such as IgE, IgG4 and IgG4/IgE ratios) in relation to the study intervention. It would be of interest to relate the results of BAT directly with the other immunologic parameters.

The compared analyses of the results of BAT at the two time-points, both at the end of the LEAP and at the end of LEAP-On studies, will help to describe immunologically whether early peanut consumption induces transient desensitisation (in which case an increase in basophil reactivity over time will be seen, with a shift in the dose-response to the left towards lower concentrations of peanut between v60 – v72) or long-term oral tolerance (in which case, there will be no significant change in the results of BAT between the two time-points) - Figure 8.1.

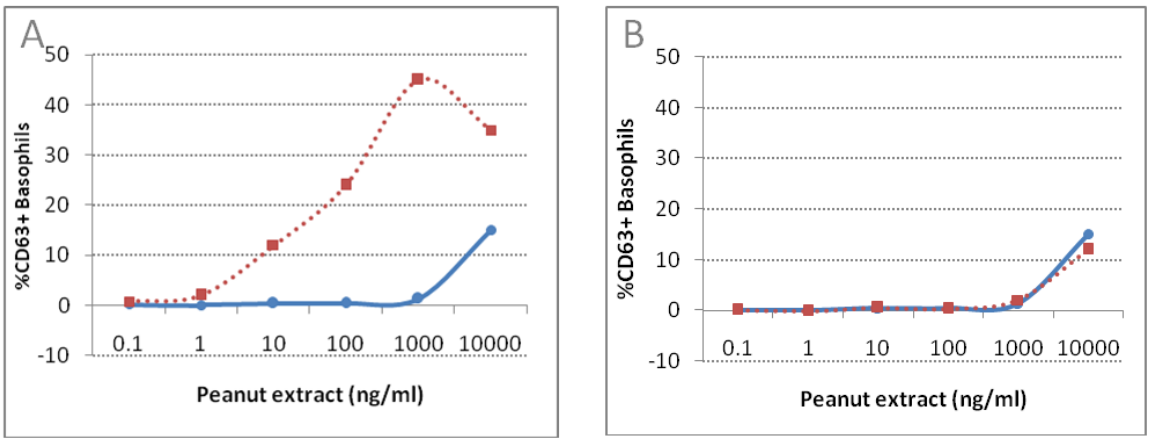

Figure 8.1 Basophil activation test in the LEAP and LEAP-On studies.

Examples of hypothetical peanut dose-response curves of CD63 expression in patients in the LEAP (continuous blue line) and LEAP-On (discontinuous red line) in case early introduction of peanut induces (A) transient desensitisation or (B) long-term tolerance.

8.6.2 Validation of other tests for the diagnosis of peanut and other food allergies

A novel mast cell degranulation assay using the LAD2 mast cell line was developed, where cells were sensitised with plasma from PA, PS or NA patients and the expression of activation markers following stimulation with allergen was assessed by flow cytometry. This experimental system reflected *in vitro* the clinical reactivity or tolerance to peanut and reproduced what happened when basophils in patients' whole blood are stimulated with peanut proteins. The mast cell activation test (MAT) may be valuable in the diagnosis of peanut allergy. I aim to test the samples of the patients recruited for the BAT study to further validate this potential diagnostic test. I will define diagnostic cut-offs for MAT to peanut and relate the results of MAT to the results previously obtained for BAT. The MAT has important additional value to BAT both for the diagnosis of food allergy and for mechanistic studies. For diagnosis, the MAT has the advantage of not requiring the use of fresh blood samples and thus allowing testing patients whose samples were collected at centres located far from the laboratory as well as testing in parallel samples that were collected at different time points. For mechanistic studies, the passive sensitisation design allows to study the functionality of antibodies and other serological factors mediating effector cell response.

The use of the basophil activation test as a diagnostic test for peanut allergy can be applied to other food allergies, namely cow's milk, egg, seed and tree nut allergies. The performance of these tests will be compared to oral food challenge (the gold-standard) and specific diagnostic cut-off values will be generated for each food. I anticipate that the BAT will have diagnostic value and will enable to reduce the need for oral food challenges to accurately diagnose these food allergies. Similarly, a MAT could be developed to other food allergens in parallel with the validation of BAT.

BAT and MAT may be applied to future immunomodulatory studies in patients with existing peanut allergy as a biomarker for clinical improvement, namely in patients undergoing allergen-specific immunotherapy (e.g. oral, sublingual, and epicutaneous) and other treatments such as omalizumab or herbal formulas.

8.6.3 Improving the management of peanut allergy

The BAT informed about the severity and the threshold of allergic reactions to peanut: patients with severe symptoms on oral food challenge had higher basophil reactivity (as measured by the percentage of CD63-positive basophils in response to peanut and to anti-IgE) and patients who reacted to lower doses of peanut on oral food challenge had higher basophil sensitivity (as measured by a lower effective dose of allergen on the BAT).The use of the BAT to predict the severity and the threshold of allergic reactions to peanut and to other foods requires validation in other patient populations before it can be translated to clinical practice.

Future studies on the stability of BAT over time and during different periods of the year (e.g. hay fever season, asthma exacerbations) would also be informative**.** While BAT parameters may not completely distinguish between those patients with severe reactions who respond to a low threshold of reactivity and those with milder reactions and a higher threshold dose, they may prove to be far more accurate in discriminating changes in the clinical threshold and severity in the same individual over time. It has already been shown that BAT to peanut, egg and milk decreases in patients who have undergone oral immunotherapy[110, 111, 232]. It would be of great value to look at the stability of BAT over time in untreated patients and at the change in BAT parameters in patients undergoing oral immunotherapy to foods and comparing this to post-treatment challenge outcome measures.

8.6.4 Further unravelling the mechanisms of peanut allergy and tolerance

A complete understanding of the mechanisms by which IgE and allergen may or may not elicit effector cell activation, that is ultimately responsible for clinical manifestations of allergic disease, requires a molecular approach. A combination of specificity, diversity and affinity of allergen-specific IgE antibodies seems to determine allergen-induced basophil activation. IgE of PA and PS patients may recognise different peanut allergen epitopes, with different location, which may have different numbers of repeats (in oligomeric allergens) and/or combinations in the allergen molecule. This could result in effector cell activation in the case of PA and not in the case of PS patients. PS patients have higher relative titres of IgG4 antibodies compared to IgE and their IgE and IgG4 epitopes match, enabling an efficient blocking effect. The affinity of IgE and IgG4 for the allergens may also be important.

In future studies, I aim to identify and compare IgE and IgG4 epitopes on peanut allergens by testing existing plasma samples from PA and PS patients in this cohort on a microarray of linear synthetic overlapping peptides covering the complete protein sequences of all known peanut allergens. Using molecular modelling methods, I will determine the location of specific IgE and IgG4 epitopes in the known three-dimensional structure of the allergen molecules and will compare the epitopes recognised by IgE and IgG4 antibodies between PA and PS patients. Finally, I will determine the clinical relevance of these epitopes experimentally using peanut allergens mutated at specific sites and a combination of well-characterised monoclonal recombinant IgE and IgG4 antibodies on the basophil and mast cell assays.

Allergen-specific monoclonal IgE and IgG4 antibodies can be generated from blood samples by single B cell cloning[151]. Thus, peanut-specific monoclonal antibodies can be engineered using blood samples of PA and PS patients. Allergen-specific monoclonal IgG4 antibodies can also be generated from monoclonal IgE antibodies by modification of the DNA encoding for the constant region of the heavy chain that is assembled into an expression vector using a published method developed by investigators at King's College London with whom I have collaborated[233]. Testing various combinations of monoclonal peanut-specific IgE antibodies generated from blood cells of PA or PS patients with known epitope specificity, affinity and clonality, in the mast cell activation assay will allow me to understand how the characteristics of the antibodies change effector cell activation. Similarly, testing monoclonal peanut-specific IgG4 antibodies generated from monoclonal peanut-specific IgE antibodies or from blood cells of PS patients in the inhibition of mast cell activation assay will allow me to understand the mechanism by which IgG4 inhibits IgE and how the various characteristics of the IgG4 antibodies can modify IgE inhibition.

IgG4 can inhibit the IgE-allergen interaction either by competition for binding to the allergen and/or by co-cross-linking of IgE and IgG4 on the surface of effector cells and consequent elicitation of the inhibitory signalling downstream the FcγRII and the FcεRI receptors. The clarification of the mechanism by which IgG4 inhibits IgE would require inhibition of basophil or mast cell activation assays with a combination of removal of plasma and blockage of FcγRII receptors before stimulation with allergen with simultaneous analyses of the expression of activation markers and intracellular activatory and inhibitory signalling molecules, such as Syk and SHIP, for example. If the IgG4 mechanism was purely competition with IgE for binding to the allergen, effector cell activation would be higher when the plasma is removed, blockage of FcγRII

would not make any difference and there would be no significant phosphorylation of SHIP following stimulation with allergen. On the contrary, if the mechanism of IgG4 consists on inhibition of the intracellular signalling pathway downstream the FcεRI receptor as a result of co-cross-linking, removal of the plasma would not make a difference in effector cell activation whereas blockage of FcγRII potentiates this activation and there is increased phosphorylation of SHIP following stimulation with allergen. There is also the possibility that both mechanisms coexist, i.e. that competition with IgE for binding to the allergen and intracellular inhibition happen simultaneously. In this case, the results of the described hypothetical experiments described (i.e. removal of plasma, blockage of FcγRII and intracellular signalling) would be a combination of the expected results for each of the possible mechanisms, as described above.

8.7 Final conclusions

- The basophil activation test diagnosed peanut allergy with 97% accuracy (98% sensitivity, 96% specificity, 95% positive predictive value and 98% negative predictive value) and allowed a reduction in the number of oral food challenges of two thirds (66%).
- In an independent population, prospectively recruited for external validation, the basophil activation test had 100% specificity, 83% sensitivity, 100% positive predictive value and 90% negative predictive value in the diagnosis of peanut allergy.
- The basophil activation test proved particularly useful in cases where conventional allergy tests failed to diagnose peanut allergy and would otherwise need an oral food challenge to clarify their allergic status. Used as a second step in the diagnostic process and performed only in selected cases, the basophil activation test maintained a high diagnostic accuracy (95%) and further reduced the required number of oral food challenges by 97%.
- The basophil activation test can be used as an *in vitro* surrogate for oral food challenges to estimate the severity and the threshold of allergic reactions to peanut and improve the management of patients with peanut allergy.
- Patients with severe reactions had a higher proportion of activated basophils in the basophil activation test than patients with milder symptoms. The dose of peanut protein to which the basophils reacted in the basophil activation test was associated with the dose at which patients reacted on oral food challenges. This information should be interpreted in light of the presence of other risk factors and can help identifying high-risk patients who require closer monitoring and intensified education.
- The basophil activation test may also be useful as a sensitive method to detect the presence of allergens and its biological activity in complex mixtures, such as foods or environmental dust samples.
- The basophil activation test may also be useful to test the function of auto-antibodies directed to IgE or its high affinity receptor.
- Although immediate-type food allergy is an IgE-mediated disease, there is a great discrepancy between the levels of allergen-specific IgE and clinical allergy. IgE levels to individual peanut allergens could not explain the discrepancy between peanut allergy and peanut sensitisation as there was a large overlap between peanut allergic and peanut tolerant patients.

- The levels of IgG4 to peanut and to individual peanut allergens were mostly comparable between peanut allergic and peanut-sensitised but tolerant children. However, the ratio of specific IgG4 to IgE was significantly higher in peanut-sensitised but tolerant compared to peanut allergic children, suggesting that the excess of IgG4 specific for peanut allergens may play a role in the absence of effector cell response characteristic of peanut tolerance.
- Plasma samples from peanut-sensitised but tolerant children inhibited peanut-induced activation of basophils and mast cells sensitised with plasma from peanut allergic patients. Depletion of IgG4 antibodies from plasma samples of peanut-sensitised but tolerant patients with IgE directed to the major peanut allergens partially restored mast cell activation, supporting the hypothesis that IgG4 plays a role in IgE inhibition in tolerant but sensitised patients.
- An improved understanding of the immune mechanisms underlying the dysregulation of oral tolerance in allergic patients and particularly of the mechanisms underlying its regulation in patients that are tolerant despite allergen-specific IgE will contribute to definitive treatment and prevention strategies for food allergy, particularly in children. For example, it could lead to the development of hypoallergenic allergen molecules with retained immunogenicity and modified IgE epitopes that could be safely and effectively used for immunotherapy, or to the development of epitope-specific IgG4 monoclonal antibodies that could be used to treat allergy or even to prevent the development of allergy in sensitised patients. The known anti-inflammatory properties of IgG4[234] make it a particularly interesting vehicle for a biological treatment for peanut and other food allergies in the future.

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Annexes

1. Patient information sheet for parents/guardians

Parent/Guardian Information Sheet

Improving the diagnosis and our understanding of peanut allergy

Research project: Diagnostic markers of clinical allergy versus sensitisation to peanut

Your child is being invited to participate in a research study that will examine the way his/her immune system responds to peanut. This research study is being conducted by Professor Gideon Lack and sponsored by the Medical Research Council (MRC). Before you make a decision on whether you wish your child to take part, it is important for you to understand why the research is being carried out and what it will involve. Please take your time to read the following information carefully and discuss with others if you wish. Please ask us if anything is unclear or if you would like more information. Take time to decide whether or not you would like your child to take part.

The purpose of this Informed Consent and Authorization Document is to tell you about the nature of the samples being collected and the additional research that may be conducted using these samples so that you make an informed decision whether you would like your child to participate. It will also inform you of how your child's personal health information may be used or given to others during the research and after the research is finished. Your decision to participate or not to participate in this research will not influence the medical care your child receives from your doctor.

What is the purpose of the study?

The purpose of the study is to clarify the mechanisms of allergy and tolerance among children with positive allergy tests to peanut and to better understand why some children with positive allergy tests react when exposed to peanut while others do not. Oral peanut challenges are often needed when the results of allergy tests are confusing and consist of giving the child peanut to eat to confirm whether or not they develop an allergic reaction. With this study, we may improve the diagnosis of peanut allergy without the need for oral peanut challenges. Moreover, this improved understanding of allergy and tolerance may lead us to new treatments to try to cure peanut and other food allergies in the future.

Why has my child been chosen?

Your child has been invited to take part because he/she may have peanut allergy, may have positive allergy tests to peanut, may have outgrown peanut allergy or may be a non-allergic control donor.

Does my child have to take part?

Your child does not have to take part in this study. You and your child are free to withdraw your consent and discontinue participation in this study at any time. If you decide not to take part, or if you decide to withdraw from the study, your decision will not affect the standard of care your child will receive at the time of his or her clinic appointment in any way.

What will happen to my child if he/she takes part?

If your child decides to take part in the study a sample of blood will be taken. This blood sample shall be collected at the same time that blood is being collected or a cannula is being inserted as part of routine medical care. The amount of blood will depend on the age and size of the child, and will be 2 teaspoons (10 ml) for children younger than 2.5 years, 4 teaspoons (20 ml) for children aged between 2.5 and 5 years, 2 tablespoons for children aged between 5 and 10 years and 3 tablespoons for children older than 10 years. Before all blood tests, a local anaesthetic cream (e.g. EMLA) or cold spray will be used on the skin to numb the area and reduce discomfort. The expected duration of the study is one visit.

What will happen with the samples when they are taken?

Once the blood samples have been taken they will be anonymised until they are destroyed. They will only be identifiable via a confidential subject ID number list which will be kept at all times in the site file which is kept in a locked office in the Division of Asthma, Allergy and Lung Biology at King's College London. The samples themselves will be stored in a locked freezer in the laboratory of the Division of Asthma, Allergy and Lung Biology at King's College London. The samples will tested in the laboratory of the Division of Asthma, Allergy and Lung Biology at King's College London and in the laboratory of the University of Kent at Medway. The samples will only be used for the tests required by his study and will only be analysed by doctors or scientists who are taking part in this study. The blood samples that are taken will be analysed to try to understand how the immune system responds to peanut in children who have IgE antibodies against peanut, as shown by positive allergy tests, and who may react or not when eating peanut. The blood samples may also be used to obtain genetic material that code for antibodies as a template for new reagents that will help improving the diagnosis and potentially creating new treatments for peanut allergy. Genetic analysis will be limited to the antibody genes only. No other genetic analysis will be performed.

What are the possible disadvantages and risks of taking part?

The risks of taking blood include temporary discomfort from the needle in your child's arm, bleeding, bruising, swelling at the needle site and, in rare instances, infection. Some people may experience light-headedness, nausea or fainting. You should contact your doctor if you or your child have any questions or your child has any side effects from participating in this study.

What are the potential benefits of taking part?

Your child may not receive any direct health benefit from taking part in this study. The information obtained from this study may aid in the improvement of our understanding about the immunologic mechanisms of peanut allergy and of the diagnosis of peanut allergy without the need for oral peanut challenges.

Will my child's taking part in the study be kept confidential?

All information collected about your child in this research will be kept strictly confidential. Research folders will be labelled as confidential and kept in a locked office at all times. Access to these folders will be restricted to study investigators and study statisticians. Any information that is stored electronically will be kept locked by password access.

What happens when the research is completed?

Once the study is completed in a few years time, the obtained information will be submitted for publication to a peer reviewed scientific or medical journal. No information that will allow the identification of any of the participants will be included in the publication.

Will my GP/health professional be informed that my child is taking part in the study?

Your GP will be informed that your child is taking part in the study in the letter sent after each clinic together with all the relevant information about your child's condition.

Who is organising and funding the research?

This study is being run by Professor Gideon Lack, Dr. Alexandra Santos and Dr. Victor Turcanu at King's College London. Funding is provided by the Medical Research Council, UK.

Who has revised the study?

The study has been reviewed and approved by St Thomas' Hospital Research Ethics Committee (REC reference 10/H0802/44) as well as by the review board of the Medical Research Council, which is supporting this project.

Will my child be paid?

Your child will not receive money or any other form of compensation for participating in this study.

Contact for further information

If you have any questions about the study and/or its procedures or safety, you may contact Professor Lack (Principal Investigator), Dr Santos (Clinical Research Fellow) or Dr Turcanu (Research Fellow) on 020 7188 0606/9730. You will be given a copy of the Patient Information Sheet and a signed copy of the Informed Consent to keep.

Termination of consent for additional research

If you decide now to agree that your child's blood can be used for further research, you can still change your mind at any time in the future. To withdraw consent, contact your doctor and withdraw your consent for the use of your child's blood for this research study. After you withdraw your consent, any blood samples for this research study that remain will be destroyed. Your physician, Professor Lack may be reached at 020 7188 9730.

2. Patient information sheet for children 7 years-old or younger

hello!!

KING'S College LONDON

welcome to our hospital.

my name is _____ and I am an **allergy doctor**.

we want to find more about **peanut allergy**.

we do **allergy tests** to find out if people are allergic to peanuts.

do you want to join in our study to find out more about peanut allergy?

it is up to you. if you do not want to, there is no problem.

what will happen if you join our study?

you will follow what is usual here at the allergy clinic.

we will put a drop on your arm skin and then make a light on the skin.
this test will not hurt you. it may be itchy.

before taking blood we will put some cream

I will talk to your mum and dad.
they will be with you all the time.

please ask me if you have any questions.
I will be happy to tell you anything you want to know.

thank you!!

3. Patient information sheet for children from 8 to 11 years-old

KING'S College LONDON

Hello!!

My name is _____ and I am an Allergy doctor.

We are running a study to know more about peanut allergy.

Why are you asking me to take part?

We are inviting you because you

- reacted to the allergy tests to peanut or

- have peanut allergy or

- did not react to your allergy tests to peanut and we wish to compare your allergy test results with other young people with peanut allergy.

We will be inviting other children to participate in our study.

Do I have to join in?

No, it is up to you. If you don't want to take part, that's ok. If you do wish to take part you can still give up at any time and no one will be upset with you.

What will happen to me if I join in?

After the allergy testing you will do the same as the other patients here at the Paediatric Allergy clinic, which will include having a small amount of blood taken from your arm as would normally happen.

Will anything in the study upset me?

The allergy testing will not hurt you. It may be a bit itchy. We will make sure that we give you some syrup that will take the itch away if you want. Taking the blood will not hurt as we will put some cream on so that you don't feel it.

4. Patient information sheet for children older than 11 years-old

Welcome to St Thomas' Hospital.

My name is _____ and I am an Allergy doctor.

My colleagues and I are performing a study to learn more about peanut allergy.

We are inviting you to participate in the study. Before you decide if you want to join in, it is very important that you understand why we are doing the study and what it will involve.

Why are we doing this study?

We want to find out more about peanut allergy and allergy testing. We wish to know why not all children with positive allergy tests to peanut develop an allergic reaction when eating peanut.

Why are you asking me to take part?

You have been invited to take part in this study because you are either allergic to peanut, reacted to the allergy test to peanut or did not react to the allergy test to peanut and we need to compare you with allergic children to better understand how allergy works. We will be inviting 100 children in total to participate in our study.

Do I have to join in?

No, it is up to you. If you don't want to take part, that's ok. If you do, you can still give up at any time and this will not affect your case in any way.

What will happen to me if I join in?

If you decide to take part in the study a sample of blood will be taken at the same time as it is being taken as part of routine medical care. Before any blood tests, we will use a cream or cold spray on the skin to numb the area and reduce discomfort. You will not have to come to the hospital for any extra visit.

Will anything in the study upset me?

The allergy testing will not hurt you, but it may be a bit itchy. We will make sure that we give you some syrup that will take the itch away if you feel that you need that.

Taking the blood will not hurt you as we will put some cream on so that you don't feel it. Sometimes people get abruise, swelling or bleeding at the needle site when taking blood but this should disappear after a short time

What are the possible benefits of joining in?

The results of this study may not help you directly, but they will help us to improve our knowledge about peanut allergy and also to find better tests to identify children that are truly peanut allergic. The blood samples will be analysed in a laboratory to see the differences in the reactions between the 4 groups of children who are participating in this study. The differences we may find will help us to know better who has true peanut allergy when we do allergy testing.

What happens when the research is finished?

Once our study is finished, we will publish our findings in a scientific paper, so that other people get to know about it.

Will any one else know that I am taking part in your study?

Your parents and the allergy team will know about the study. We will also tell your family doctor (GP).

Will any one know what will happen and what I've said?

Any information you give as well as anything happening during the study will be kept confidential. Only people doing the research will know about it.

Has someone checked that it is OK to do this study?

Before any one is allowed to do a study, it has to be approved by a group of people that include doctors, lay people and administrators to check that all participants will be treated ethically. This is called an Ethics Committee. This study was approved by St Thomas' Hospital Research Ethics Committee. This study was also checked by experts from the Medical Research Council, which is supporting the study.

Thank you for reading this. Now you can decide together with your parents if you want to take part in this study. If you decide to take part you will be given a consent form to sign, which your parents will have to sign as well.

5. Informed consent form for study participants

CONSENT FORM FOR PERSON WITH PARENTAL RESPONSIBILITY

Improving the diagnosis and our understanding of peanut allergy

Research project: Diagnostic markers of clinical allergy versus sensitisation to peanut

1. I confirm that I have read and understood the information sheet for the study entitled "Diagnostic markers of clinical allergy versus sensitisation to peanut" (08/04/2011 version 4) and have had the opportunity to ask questions ☐

2. I understand that my child's participation is voluntary and that I am free to withdraw at any time, without giving any reason, without his/her medical care or legal rights being affected ☐

3. I understand that sections of my child's medical notes may be looked at by responsible individuals from the Medical Research Council, regulatory authorities or from the NHS Trust, where it is relevant to my child taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to give samples of blood for use in the research described. I understand how the samples will be collected, that giving samples for this research is voluntary and that I am free to withdraw my approval at any time, without giving any reason, without my medical care or legal rights being affected. ☐

5. I agree that my child's DNA may be isolated from blood samples for genetic analysis ☐

6. I understand that my GP will be informed if any of the results of the medical tests done as part of the research are important for my health. ☐

7. I agree that my child may take part in the above study ☐

_____
Name of participant (print)

_____
Name of person with parental responsibility (print)

_____ _____
Signature of person with parental responsibility Date

_____
Name of person conducting informed consent discussion (print)

_____ _____
Signature of person conducting informed consent discussion Date

Diagnostic markers of clinical allergy versus sensitisation to peanut - version 3 08-04-2011 1/1

6. Patient information sheet for adult volunteers

Participant Information Sheet

Improving the diagnosis and our understanding of peanut allergy

Research project: Diagnostic markers of clinical allergy versus sensitisation to peanut

You are being invited to participate in a research study that will examine the way children's immune system respond to peanut. This research study is being conducted by Professor Gideon Lack and sponsored by the Medical Research Council (MRC). Before you make a decision on whether you wish to take part, it is important for you to understand why the research is being carried out and what it will involve. Please take your time to read the following information carefully and discuss with others if you wish. Please ask us if anything is unclear or if you would like more information. Take time to decide whether or not you would like to take part.

The purpose of this Informed Consent and Authorization Document is to tell you about the nature of the samples being collected and the additional research that may be conducted using these samples so that you make an informed decision whether you would like to participate. It will also inform you of how your personal health information may be used or given to others during the research and after the research is finished.

What is the purpose of the study?

The purpose of the study is to clarify the mechanisms of allergy and tolerance among children with positive allergy tests to peanut and to better understand why some children with positive allergy tests react when exposed to peanut while others do not. Oral peanut challenges are often needed when the results of allergy tests are confusing and consist of giving the child peanut to eat to confirm whether or not they develop an allergic reaction. With this study, we may improve the diagnosis of peanut allergy without the need for oral peanut challenges. Moreover, this improved understanding of allergy and tolerance may lead us to new treatments to try to cure peanut and other food allergies in the future.

Why have I been chosen?

We wish to take blood from a wide range of adult individuals to be able to analyse the immune response of children with positive allergy tests to peanut by doing specific immunologic assays. The reason why adult blood is needed is related to the fact that we are studying a rare population of cells that circulate in the blood. To study them we need a volume of blood that we cannot collect from small children. You have been invited to take part because you are a healthy adult and we would therefore welcome your participation in our study.

Do I have to take part?

It is up to you whether or not to take part. If you do decide to take part you will be given this information sheet to keep and asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

What will happen if I take part?

You have been asked to give blood for research purposes. An experienced member of staff will take between 4 teaspoons and 4 table spoons (i.e. between 20 and 100ml) of venous blood in any single occasion. You may decide to donate blood in more than one occasion for the same research study. We will adhere strictly to the guidelines adopted by the National Blood Transfusion Service who take a maximum of 470ml every 16 weeks. Donors who give blood regularly will be tested for haemoglobin levels (males every 6 months and females every 3 months) to monitor for anaemia.

What will happen with the samples when they are taken?

Once the blood samples have been taken they will be anonymised until they are destroyed. They will only be identifiable via a confidential subject ID number list which will be kept at all times in the site file which is kept in a locked office in the Division of Asthma, Allergy and Lung Biology at King's College London. The samples themselves will be stored in a locked freezer in the laboratory of the Division of Asthma, Allergy and Lung Biology at King's College London. The samples will be processed in the laboratory of the Division of Asthma, Allergy and Lung Biology at King's College London and in the laboratory of the University of Kent at Medway. The samples will only be used for the tests required by his study and will only be analysed by doctors or scientists who are taking part in this study.

What are the possible disadvantages and risks of taking part?

You may experience slight discomfort during collection of blood. In some occasions taking blood may result in temporary discomfort from the needle in your arm, bleeding, bruising, swelling at the needle site and, in rare instances, infection. Some people may experience light-headedness, nausea or fainting. You should contact the research team if you have any questions or if you have any side effects from participating in this study.

What are the potential benefits of taking part?

You will not receive any direct health benefit from taking part in this study. The information obtained from this study may aid in the improvement of our understanding about the immunologic mechanisms of peanut allergy and of the diagnosis of peanut allergy without the need for oral peanut challenges.

Who is organising and funding the research?

This study is being run by Professor Gideon Lack, Dr. Alexandra Santos and Dr. Victor Turcanu at King's College London. Funding is provided by the Medical Research Council, UK.

Who has revised the study?

The study has been reviewed and approved by St Thomas' Hospital Research Ethics Committee (REC reference 10/H0802/44) as well as by the review board of the Medical Research Council, which is supporting this project.

Will I be paid?

You will not receive any money for participating in this study, but travel expenses may be reimbursed.

Contact for further information

If you have any questions about the study and/or its procedures or safety, you may contact Professor Lack (Principal Investigator), Dr Santos (Clinical Research Fellow) or Dr Turcanu (Research Fellow) on 020 7188 0606/9730. You will be given a copy of the Patient Information Sheet and a signed copy of the Informed Consent to keep.

Thank you for considering taking part in this study.

7. Informed consent form for adult volunteers

CONSENT FORM

Improving the diagnosis and our understanding of peanut allergy

Research project: Diagnostic markers of clinical allergy versus sensitisation to peanut

1. I confirm that I have read and understood the information sheet for the study entitled "Diagnostic markers of clinical allergy versus sensitisation to peanut" (08/04/2011 version 2) and have had the opportunity to ask questions ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected ☐

3. I understand that sections of my medical notes may be looked at by responsible individuals from the Medical Research Council, regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to give samples of blood for use in the research described. I understand how the samples will be collected, that giving samples for this research is voluntary and that I am free to withdraw my approval at any time, without giving any reason, without my medical care or legal rights being affected. ☐

5. I agree to take part in the above study ☐

_____
Name of participant (print)

_____ _____
Signature of participant Date

_____
Name of person conducting informed consent discussion (print)

_____ _____
Signature of person conducting informed consent discussion Date

8. Poster to recruit adult volunteers

Would you like to help us with our research into Peanut Allergy *and* find out about your allergy status?

KING'S College LONDON

Are you

- **Aged 18 or over ?**
- **Healthy ?**
- **Or suffering from allergies ?**

We will perform a skin test to see whether you are allergic and we will do a blood test (20-100mls, up to 6 tablespoons)

For more information please contact:

Alexandra Santos
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Fax: +44 (0) 20 7403 8640
Email: alexandra.santos@kcl.ac.uk

This research has been reviewed and approved by the St Thomas' Research Ethics Committe

MRC & Asthma UK Centre in Allergic Mechanisms of Asthma

MRC Medical Research Council | KING'S College LONDON | Imperial College London | asthma UK

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